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**Determination of gelation mechanism and prevention methods of frozen-thawed hen egg yolk**

by

**Monica Primacella**

A thesis submitted to the graduate faculty  
in partial fulfillment of the requirements for the degree of  
**MASTER OF SCIENCE**

Major: Food Science and Technology

Program of Study Committee:  
Nuria Acevedo, Co-major Professor  
Tong Wang, Co-major Professor  
Buddhi Lamsal

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2017

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**NOMENCLATURE**

CMC	Carboxymethyl cellulose
DSC	Differential scanning calorimetry
LDL	Low-density lipoproteins
LVR	Linear viscoelastic region
HDL	High-density lipoprotein
HCMC	Hydrolyzed carboxymethyl cellulose
HEW	Hydrolyzed egg white
HEY	Hydrolyzed egg yolk
MW	Molecular weight
PEG	Polyethylene glycol
RI	Refractive index
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
So	Protein surface hydrophobicity
TW	Tween-80



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**ABSTRACT**

Gelation of egg yolk during freezing and thawing has been a hurdle to food processors for decades, because of the reduced yolk functionality. Many studies have been performed in the past to understand the mechanism of gelation. However, this concept has not been fully elucidated. Currently, industry practices the addition of 10% salt or sugar to inhibit gelation. Although gelation is inhibited, this practice causes a significant change in flavor, which then limits the yolk application. It is therefore our objective to further study the gelation mechanism and identify the component(s) responsible for gelation through analyzing fractional mass distribution, protein distribution and rheological properties in four reconstituted yolk systems, so that alternative gelation prevention methods could be determined. Protein aggregation occurred as five weeks of freezing was induced in four recombined yolk systems containing different proportions of plasma and granule. All four frozen-thawed yolk systems had a significant increase ( $p < 0.05$ ) in the mass of granule fraction and an additional layer of floating lipidic fraction. Gel strength was shown to increase with increasing granule content in the system. This study showed that aggregations involved heterogeneous interactions between plasma and granule components, including LDL, HDL, and  $\alpha$ -livetins. Ingredients that could reduce the degree of gelation were identified. Hydrolyzed carboxymethyl cellulose (HCCM), hydrolyzed egg white and yolk (HEW and HEY), and proline were able to significantly reduce ( $p < 0.05$ ) the hardness of the frozen-thawed yolk mixtures. The gelation-inhibiting mechanisms of these additives were assessed through differential scanning calorimetry (DSC), particle size distribution, and protein surface hydrophobicity as compared to salt and sugar.

## **CHAPTER 1. GENERAL INTRODUCTION**

Hen egg yolks are used extensively in many food products because of their nutritional, organoleptic and functional properties. Industrially, yolk products are produced following the breaking of eggs, and these include liquid, dried and frozen egg yolks. Freezing is commonly preferred in many food products, because it provides ease of transport and storage, extends shelf life, while causing minimal damage to the food quality. However, when egg yolks are frozen below  $-6^{\circ}\text{C}$ , an irreversible change in fluidity, known as gelation, occurs (Cotterill, 1986a; Moran, 1925). Gelation is undesirable because it reduces the yolk functionality, including its ability to disperse in water (Powrie, Little, & Lopez, 1963). Egg processing industry currently practices the addition of salt or sugar to yolk prior to freezing to prevent gelation. Although gelation is inhibited, this approach results in limited applications of the yolk due to the change in flavor. Alternative methods are yet to be explored following better elucidation of yolk gelation mechanism induced by freezing and thawing.

### **Thesis Organization**

This thesis begins with a review of literature focusing on the gelation of hen egg yolk during freezing and thawing, and other relevant information such as yolk production, composition, nutrition and functionality in food products. Two manuscripts are included in this thesis following the literature review. Manuscript authors are part of the Department of Food Science and Human Nutrition. Drs. Nuria Acevedo and Tong Wang are the corresponding authors for the first and second manuscript, respectively. A general conclusion and appendix conclude this thesis.

## **Literature Review**

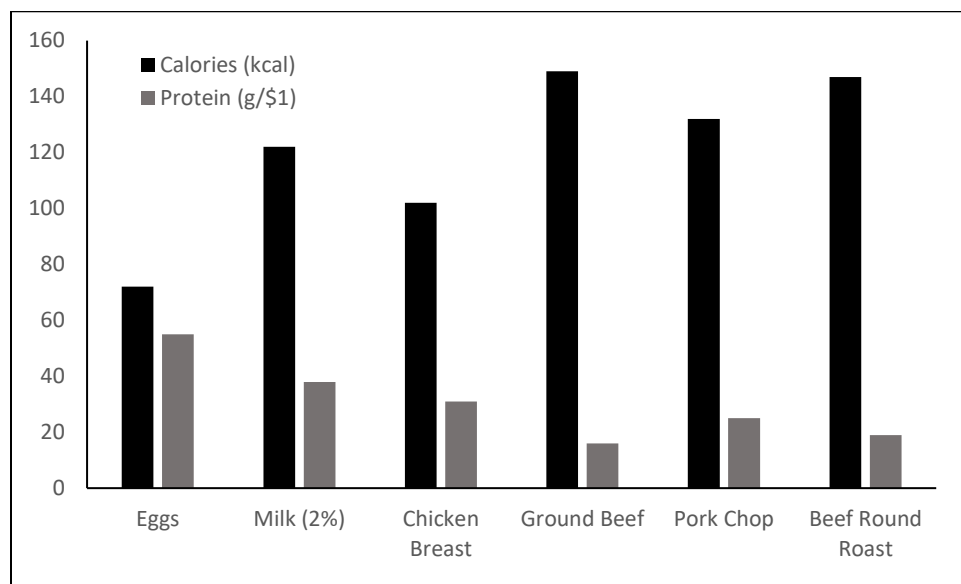
### **Egg production and consumption**

The United States is the second largest egg producing country in the world, with majority of eggs produced in Iowa, Ohio, Indiana, Pennsylvania and Texas. These states represent approximately 52% of all U.S. hens (USDA, 2017). Out of 245.54 million cases of table eggs produced in 2016, about 58.6% of the eggs went to retail, 8% were used by food service industry, 2.3% were exported, and about 31.1% (or 76.33 million cases) were broken for further processing (AEB, 2017b).

The egg production process involves several phases, including laying, collecting, washing, candling, grading, sorting and packing, shipping, selling and storing. Eggs are classified based on the interior and exterior quality at the time they are packed, and are assigned grade AA, A or B. There is no difference in the nutritional value between grades, but eggs sold at the retail level must meet the standards for Grade B or better. The grading covers the firmness and thickness of the white as well as the roundness and elevation of the yolk. Most of the eggs in the U.S. reach the grocery store in 1-3 days after being laid, and have to remain refrigerated unlike in several countries where refrigeration is optional. This is because the U.S. regulation requires eggs to be washed and sanitized, and therefore the bloom or the natural coating to protect porous shell is lost (AEB, 2017c).

Eggs remained as a desirable commodity to the general public. With the removal of daily cholesterol intake on the 2015-2020 Dietary Guidelines for Americans (DGA) (AEB, 2017a) and encouragement to include regular consumption of eggs along with other nutrient-rich foods (Kanter, 2016), the growth of the egg industry is expected to continue.

Eggs are a nutrient dense, low-calorie, and cost-effective food. One large egg contains 70 calories and varying amounts of thirteen essential vitamins and minerals. It also contains 6 g of high-quality protein and all nine essential amino acids. Its valuable compositions make eggs an excellent source of choline and selenium, and a good source of protein, vitamin D, vitamin B12, phosphorus and riboflavin (ENC, 2017). Compared to other protein-rich food, eggs provides higher protein value for each \$1 spent, and relatively fewer calories (Figure 1.1).



**Figure 1.1.** Comparison of calorie and protein content in protein-rich foods

In addition to the nutritional and organoleptic properties, hen eggs are widely used in the food industry because of their multifunctional properties, such as foaming, gelling, and emulsifying. With the continuing demand for more convenience products and advances in egg-processing technology, there has been a continuing growth of further processed egg products. In fact, approximately 30% of the total consumption of eggs is in the form of further processed egg products, including liquid, dried, and frozen whole eggs, yolks and whites (Froning, 2008).

## **Egg products processing**

Egg products are used widely in foodservice industry and commercial food industry, due to convenience, ease in handling and storing, and higher level of food safety. The term “egg products” refer to egg that are removed from their shells at facilities known as “breaker plants” (USDA, 2011). As mandated by the Egg Product Inspection Act (EPIA), the plant has to be under regular USDA inspection. The USDA’s Food Safety and Inspection Service (FSIS) has the authority to provide insight if the freshly laid eggs are to be placed in cartons for consumers or sent to an egg processing facility (USDA, 2011).

Prior to breaking, eggs must be washed and completely dried. The breaking process involves a cracker to crack the shells at the center and pull the two shells apart, and a yolk-albumen separator, which consists of two cups placed one above the other. The yolk remains in the top cup while the albumen slides to the bottom cup. The liquid egg products are filtered, added with ingredients (salt, sugar, etc.), blended, standardized, and pasteurized. Upon pasteurization, egg products are either sent to packaging facility, or frozen/dried (Wu, 2014).

## **Processed yolk products**

### **Liquid egg yolk**

The solid content of pure egg yolk is about  $51.9 \pm 0.1\%$ . Egg yolk produced by egg-breaking machines contains 46-48% solids depending on the amount of egg yolk adhering to the yolk (Cotterill, 1986a). Liquid egg yolk product without additives is usually standardized to 43-44% by the addition of egg white to prevent transitional change in the viscosity of the egg yolk (Cunningham, 1972). Since commercial egg yolk contains egg white, its pH level is slightly higher than 6.0.

### **Frozen egg yolk**

About 30% of the total liquid egg production is frozen (Cotterill, 1986b). While freezing causes large reductions in bacterial counts, major changes in texture occur, especially in products containing egg yolk. Gelation in egg yolk, which occurs following freezing and thawing, can be easily controlled through the addition of 10% salt or sugar.

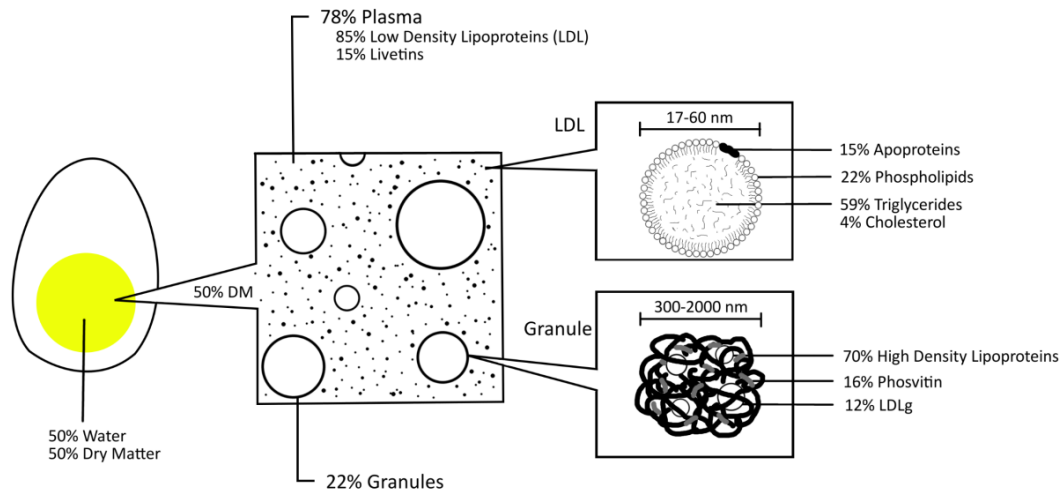
### **Dried egg yolk**

Dried egg products has some advantages over liquid and frozen egg products, such as lower storage and transportation cost, ease of handling in a sanitary manner, good uniformity, more control of water content during formulation of food products (Bergquist, 1986). Dried egg yolk can be produced by spray drying, pan drying or freeze-drying. To produce room temperature shelf-stable product, glucose is removed from the liquid or converted to an acid before drying. The dried products do not have food whipping ability, but are excellent in binding, emulsifying, and heat-coagulating properties (Bergquist, 1986). Additives such as salt and sugar are sometimes added to preserve the yolk functionalities that might be lost during processing involving extreme temperatures.

### **Egg yolk composition**

An average liquid whole egg consists of 64% white (albumen) and 36% yolk (Figure 1.2). The albumen consists of 12% dry matter, which is composed predominantly of protein with small amounts of minerals and sugars and trace amount of fat. On the other hand, the yolk consists of 50% dry matter with about 70% fat and 30% protein (Brooks & Taylor, 1955; Romanoff & Romanoff, 1949).

In native conditions, yolk is a complex assembly of lipids and proteins, which can be separated into two major fractions: soluble plasma and the non-soluble protein aggregates (granules). Plasma corresponds to about 75-81% of the yolk dry matter and is mainly constituted of 85% low density lipoproteins (LDLs) and 15% livetins, whereas granules accounts to about 19-25% of yolk dry matter, and consists of 70% high density lipoproteins (HDLs), 16% phosvitin, and 12% LDLs (Burley & Cook, 1961). Plasma gathers 85% of the phospholipids in yolk and 52-59% of the proteins, whereas granules contain 15% phospholipids and 42-48% proteins (Saari, Powrie, & Fennema, 1964). The plasma and granule fractions can be obtained by dilution and mild centrifugation (McBee & Cotterill, 1979).



**Figure 1.2.** Composition of egg yolk (Au, 2015)

### Yolk component structures and properties

LDLs are the main constituent of yolk, representing 2/3 of the yolk dry matter. LDLs are described as spherical nanoparticles (17-60 nm) with a lipid core of liquid state triglycerides and cholesterol esters surrounded by phospholipid and protein monofilm, referred to as apoprotein (Cook & Martin, 1969). Phospholipids maintain the stability of the LDL structure and some



cholesterols located on the outer monofilm helps to add rigidity to the structure (Burley, 1975). At least 6 different apoproteins comprise the LDL particle, where their pI range from 6.3 to 7.5. Altogether LDL account for of 11-17% protein and 83-89% lipid, which could be broken down to 69% triglycerides, 27% phospholipids and 4% cholesterol and cholesterol esters (Cook & Martin, 1969). LDL solubility in aqueous solutions is independent of pH and ionic conditions due to their low density of 0.982 (Anton, 2013).

Granules consist of protein aggregates ranging in diameter from 0.3 to 2  $\mu\text{m}$ , depending on the environmental conditions (Chang, Powrie, & Fennema, 1977). At pH 4.3 to 6.5, granules form HDL-phosvitin complexes linked by phosphocalcic bridges due to the high content of calcium-binding phosphoserine amino acids. The phosphocalcic bridges make granules very compact, poorly hydrated, and weakly accessible to enzymes. Consequently, granules are more resistant than plasma against thermal denaturation and heat gelation (Castellani, Guérin-Dubiard, David-Briand, & Anton, 2004; Causeret, Matringe, & Lorient, 1991). It was reported that the emulsifying activity of plasma dropped after heating at 72°C, while it remained steady for granules (Le Denmat, Anton, & Gandemer, 1999).

The phosphocalcic bridges are disrupted when ionic strength is higher than 0.3 M NaCl due to the displacement of the divalent calcium by the monovalent sodium. At this condition, granules are 80% soluble because phosvitin is a soluble protein and HDL behaves like soluble proteins. At 1.71 M NaCl, complete dissociation of granules occurs (Chang, Powrie, & Fennema, 1977).

### **Egg yolk gelation induced by freezing and thawing**

Yolk gelation caused by freezing and thawing was first studied in 1925 by Moran. He found that an egg yolk has a freezing point of  $-0.65^{\circ}\text{C}$  and when it is kept below  $-6^{\circ}\text{C}$ , a detrimental change in fluidity occurs where the liquid yolk transitioned into “a stiff paste-like putty” that is difficult to mix with other ingredients (Moran, 1925). However, this did not occur when the yolk was rapidly cooled to as low as  $-11^{\circ}\text{C}$ , indicating that ice crystal formation is necessary for gelation to occur. According to Riedel (1972), 81% of the water content of egg yolk are crystallized at  $-6^{\circ}\text{C}$ , suggesting that this is the amount of crystals needed to induce gelation. Jaax and Travnicek (1968) and Mahadevan, Satyanarayana and Kumar (1969) agreed with this finding that slow cooling is needed to induce gelation, as rapid cooling even with extended storage in low temperatures (33-49 days in  $-20^{\circ}\text{C}$ ) did not result in gelation.

Several factors, including frozen storage time and temperature (Au, Acevedo, Horner, & Wang, 2015; Powrie, Little, & Lopez, 1963; Wakamatu, Saito, & Sato, 1981) and freezing and thawing rate (Lopez, Fellers, & Powrie, 1954; Powrie, 1968), that have influence on the rate and size of ice crystal formation, dehydration of proteins, and concentrations effects, are known to affect the degree of gelation. The rate of gelation was found to increase when the storage temperature was lowered from  $-10^{\circ}\text{C}$  to  $-14^{\circ}\text{C}$ . Yolk regained its normal fluidity when supercooled in liquid nitrogen at  $-11^{\circ}\text{C}$  for 7 days and thawed rapidly in mercury at  $30^{\circ}\text{C}$ . Although factors affecting gelation are known, the mechanism of yolk freeze-thaw gelation has not been fully elucidated.

Many studies have shown that LDL participates in yolk gelation (Powrie, Little, & Lopez, 1963; Saari, Powrie, & Fennema, 1964; Sato & Takagaki, 1976; Wakamatu, Sato, & Saito, 1982). Moran (1925) suggested that concentrated salts from water removal through ice

crystal formation might cause lipoprotein precipitation. Soliman and Van den Berg (1971) added that pH change in the unfrozen phase might also cause LDL aggregation in addition to salt concentration. Hasiak, Vadehra, Baker and Hood (1972) found that freezing induced LDL aggregation through the alteration of water structure. Powrie, Little and Lopez (1963) and Meyer and Woodburn (1965) found that LDL of frozen-thawed yolk showed loss of electrophoretic mobility when compared to those of fresh yolk. Reinke (1967) suggested that phosphatidylcholine and/or calcium act as bridging components between aggregated proteins in gelled yolk. Saari, Powrie and Fennema (1964) and Mahadevan, Satyanarayana and Kumar (1969) found that the LDL-rich plasma fraction gelled upon freezing and thawing, and the gelation could be inhibited by the same compounds used to inhibit yolk gelation. This suggested that plasma components must be involved in gelation.

However, it remained unclear how LDL aggregated and different proposed mechanisms exist. According to Holdsworth and Finean (1972) and Kurisaki, Kaminogawa and Yamauchi (1980), disruption and removal of the surface layer of LDL is the first step of aggregation. Wakamatu and Sato (1980) believed that LDL aggregation is attributed to conformational changes of LDL. Telis and Kieckbusch (1997) proposed that breaking of LDL micelle followed by dehydration during freezing leads to aggregation.

Participation of other yolk components in yolk gel formation has also been suggested. Powrie (1968) and Chang, Powrie and Fennema (1977) proposed that some granule components might have been liberated during freezing due to the increased salt concentration following water crystal formation, and could have interacted with plasma LDL and promote gelation. This assumption was made following investigation of differences in gel properties between samples containing yolk and plasma only. Through paper electrophoresis, it was shown that after gelation

the migration of LDL bands from the yolk sample was restricted and some livetin bands were not visible, suggesting aggregation between LDL micelles and livetins. The reduction in LDL mobility was not as extensive in the plasma sample suggesting granule participation in enhancing gelation.

The most recent study on yolk freeze-thawed gelation also showed that both constituents of plasma and granules contribute to gelation, as shown through PAGE and particle size analyses (Au, Acevedo, Horner, & Wang, 2015). It was shown through increasing gel strength and particle size, as well as decrease in water and lipid-water mobility that aggregation of lipoproteins occurred as storage time continued. However, after 84 days of storage, protein and lipid mobility as well as gel strength increased and small particles were detected. This suggested the liberation of protein or lipoprotein from previously formed aggregates and further aggregation of these constituents.

While a few studies have suggested that gelation involves other components other than plasma LDL, most studies done on egg yolk gelation during freezing and thawing only included plasma LDL. The whole yolk components need to be incorporated in the experiment design to better elucidate the mechanism of egg yolk gelation. The ways in which granule components participate in yolk gelation still needs further validation.

### **Yolk gelation prevention**

Yolk gelation can be inhibited by mechanical and chemical treatments prior to freezing. Homogenization and colloid milling were found to have some effects in minimizing gelation (Lopez, Fellers, & Powrie, 1954; Pearce & Lavers, 1949). The first reported chemical agent for gelation inhibition was sucrose (Moran, 1925), where the incorporation of 10% sucrose in yolk

resulted in no considerable changes in the yolk fluidity following freezing at  $-11^{\circ}\text{C}$  and thawing. Powrie, Little and Lopez (1963) suggested that the protective effect of sucrose might be attributed to inhibition of denaturation of aggregation of the yolk proteins.

Other cryoprotective compounds such as some other sugars, some salts, and glycerol, as well as some proteolytic enzymes have also been reported successful in inhibiting gelation (Lesser, 1948; Lopez, Fellers, & Powrie, 1954; Lopez, Fellers, & Powrie, 1955; Thomas & Bailey, 1933; Tressler, 1932). Lopez, Fellers and Powrie (1954) studied the gelation inhibition ability of arabinose, galactose, cellobiose, lactose, maltose, raffinose, sucrose and dextrose. While the yolk treated with 10% arabinose and galactose had lower degree of gelation compared to the yolk treated with 10% sucrose or dextrose, the rest of the treatments did not show any inhibitory effect on gelation. This suggested that sugars do not inhibit gelation merely by lowering the freezing point of water in yolk.

It was proposed that gelation is related to changes involving the lipoproteins of yolk following the removal of water. Gelation inhibition through addition of enzyme supported this theory, as enzyme can attack HDL and LDL to form lysophospholipoprotein complexes with altered solubility. Feeney, MacDonnell and Fraenkel-Conrat (1954) found that yolk treated with crotoxin (lecithinase A) at 1mg/mL yolk and 10mg/mL yolk had significantly lower degree (10-20%) of gelation compared to untreated yolk. According to Lopez, Fellers and Powrie (1955), the most effective proteolytic treatment was the addition of 0.05% papain to yolk under incubation period of 15-20 minutes at  $25^{\circ}\text{C}$ . Papain was better compared to other proteolytic enzymes because it did not seriously affect the organoleptic properties.

Cysteine was observed to reduce but not inhibit gelation (Meyer & Woodburn, 1965; Powrie, Little, & Lopez, 1963). The reduction was speculated to be caused by cysteine rupturing

the intramolecular disulfide bonds in lipoproteins, causing a partial uncoiling of the protein molecules.

Currently, the addition of 10% salt (NaCl) or sugar (sucrose) is the most common practice used in industry due to the effectiveness of gelation inhibition and relatively low cost. Telis and Kiechbusch (1998) and Wakamatu, Sato and Saito (1983) found that sucrose, glycerol and magnesium chloride can prevent yolk gelation when used at a concentration as low as 2%, and improved cryoprotectant effects were observed as the concentrations were increased. On the other hand, sodium chloride can be either a gelation inhibitor or accelerator depending on the dosage used. Sodium chloride acts as an inhibitor of LDL gelation by increasing the unfrozen water in the sample; as an accelerator, it promotes removal of water from the LDL-water-sodium chloride complex (Wakamatu, Sato, & Saito, 1983).

Homogenization and colloid milling were reported to inhibit but not prevent yolk gelation. When yolk is run through a colloid mill with a 0.003" clearance for three passes, the degree of gelation is low (Lopez, Fellers, & Powrie, 1954).

More research is needed to determine new treatments that can prevent yolk gelation while causing minimal changes to the yolk natural attributes (flavor, color, texture, etc.). The addition of 10% salt or sugar has been effective in preventing gelation. However, the flavor and texture are markedly altered. Thus, these yolk products are suitable only for certain applications. Identification of new additives that can solve this issue may broaden the range of application in which frozen yolk is used. Combinations of these additives paired with mechanical treatments, due to the different nature of work, may allow less use of additive and higher gelation reduction.

### **Hypothesis of this Research**

It is hypothesized that gelation is a result of yolk protein aggregations, caused by concentration of yolk components due to ice crystal formation during slow freezing. The change in the yolk physiochemical condition is unfavorable in maintaining the structural integrity of the yolk matrix. Water removed through the formation of ice crystals might have altered yolk protein structure, and the previously inaccessible hydrophobic region might now be exposed for protein-protein interaction. The granule component has often been overlooked when studying yolk gelation, but some studies have found that yolk gelation involve more than just the plasma LDL. The increased ion concentration can lead to disruption of the yolk granular component, leading to aggregations between the released granular HDL and LDL with the plasma components including the LDL and livetin. Therefore, treatments that can interfere with water crystal formation and growth, and prevent protein aggregation might be effective in preventing gelation in egg yolk during freezing and thawing.

### **Objective**

The overall objective of this research was to better elucidate the mechanism of egg yolk gelation induced by freezing and thawing, and to determine treatments that can inhibit gelation. Recombined yolk systems containing different proportions of plasma and granule were used to study the mechanism of gelation. Changes in protein behavior was studied through mass distribution of yolk fractions, and the changes in protein structure, such as aggregation, of each fraction was observed through sodium dodecyl sulfate polyacrylamide gel electrophoresis. Rheology was also measured to investigate the participation of granule content in gelation. The gelation mechanism was studied so that gelation inhibitors could be identified. The performance

of these inhibitors, which include new food additives and mechanical treatments, was characterized through monitoring changes in transition temperatures and amount of freezable water through differential scanning calorimetry (DSC), changes in particle size/aggregation through particle size analysis using laser diffraction and protein surface hydrophobicity using spectrofluorometry.

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## CHAPTER 2. USE OF RECONSTITUED YOLK SYSTEMS TO STUDY THE GELATION MECHANISM OF FROZEN-THAWED HEN EGG YOLK

A manuscript accepted for publication in *Journal of Agricultural and Food Chemistry*

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### Abstract

Yolk gelation upon 5-week freezing was studied in 4 recombined yolk systems containing different plasma and granule proportions. Fractionation for mass distribution, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for protein distribution and rheological properties were explored. Results indicate that both plasma and granule components including LDL, HDL, and  $\alpha$ -livetin proteins, contributed to gelation. Protein aggregation was reflected through large mass increase in granule fraction and appearance of a floating LDL layer upon fractionation of gelated yolk systems. A significant increase in gel strength (elastic modulus,  $G'$ ) was observed with the increase of granule content. Overall, this study provides a better understanding of yolk gelation mechanism that may consequently lead to the design of innovative methods for preventing gelation. A schematic presentation of yolk gelation mechanism is also proposed.

### Introduction

Hen egg yolk is one of the most used ingredients in many products due to its high nutritional value and unique functionalities. Being an excellent emulsifier, egg yolk is used

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extensively in foods, such as mayonnaise, salad dressing, and sauces. Recent statistical analysis showed that out of the 231 million cases of shell eggs (or 83 billion eggs) produced in U.S. in 2015, approximately 30% underwent breaking for further processing <sup>1</sup>. However, when egg yolks are frozen under -6°C and thawed, an irreversible loss of fluidity, termed gelation, occurs. This change is unfavorable because it reduces the yolk functionality and its ability to mix with other ingredients <sup>2</sup>. Current gelation prevention practices include the additions of salt, sugar, or corn syrup to yolk prior to freezing <sup>3</sup>. However, consumer's preference for low salt and low sugar products may limit the range of frozen yolk application.

Researchers have proposed many different explanations regarding the mechanism of gelation related to its composition. Yolk is composed of about 50% water and 50% dry matter, in which the dry matter could be broken down to 77-81% plasma and 19-23% granules. Plasma contains 85% low density lipoprotein (LDL) and 15% livetin, while granule contains 70% high density lipoprotein (HDL), 16% phosvitin, and 12% LDL <sup>4</sup>. Scientists have stated no difference between plasma LDL and granule LDL. The most common explanation for yolk gelation is that there are aggregations of plasma LDLs that result from a concentration of yolk components due to the formation of large ice crystals during freezing <sup>5-7</sup>. However, disagreement exists on the mechanism of LDL aggregation. Telis and Kiechbusch <sup>6</sup> proposed that dehydration of proteins located on the surface of LDL micelles following the breaking up of LDL micelles leads to LDL aggregation. Kurisaki et al. <sup>8</sup> suggested surface components of LDL are liberated during freeze-thaw, causing aggregation of the newly exposed sites. Wakamatu et al. <sup>7</sup> believed that LDL aggregates due to conformational changes, and not because of liberation of LDL components. Interaction of protein molecules after disruption of lecithin-protein interactions is another

proposed LDL aggregation mechanism by Mahadevan et al. <sup>9</sup> and Kumar and Mahadevan <sup>10</sup>. The real cause of LDL aggregation is still unclear.

While majority of studies emphasized the role of plasma LDL in yolk gelation, only a few studies include the granule fraction in their work. Wakamatu et al. <sup>7</sup> indicated that they could not exclude the involvement of granular LDL (LDLg) in LDL aggregation because the lipid compositions of LDL and LDLg were very similar. Chang et al. <sup>5</sup> found that gelation was enhanced when granule is in the system, compared to plasma alone. They proposed that LDLg are released from granules disrupted during freezing, causing both LDL and LDLg to aggregate. Regardless of the differences in existing proposed mechanisms of gelation, most researchers agreed that removal of water through ice crystal formation is necessary for gelation to occur.

Removal of water by freezing might have decreased physical distance and increased hydrophobic interaction which cause LDL destabilization. Studies showed that phospholipase-A treated LDL had inhibited gelation because they were more hydrophilic <sup>11, 12</sup>. A phospholipase-C treated LDL was more lipophilic and it promoted aggregation <sup>9</sup>. This provides some evidence that gelation may be due to surface hydrophobic interactions of LDL.

The most recent study on the effect of prolonged freezing storage on egg yolk gelation suggested the occurrence of two-stage gelation, which involved aggregation of lipoprotein particles resulting from water removal during slow freezing in the first stage (d 1-28), and release and re-aggregation between or within the previously aggregated proteins in the second stage (between d 28-84) forming a stronger gel network <sup>13</sup>. The author also suggested that granules/HDL particles were also involved in the aggregation, and various methods including evaluations of particle size, matrix mobility, protein aggregation and microstructure were able to

show that these components played a role in gelation. However, the involvement of HDL proteins in gelation still needs further validation.

The overall goal of this research is to elucidate how the different yolk fractions are involved in freeze-thawed yolk gelation. We hypothesize that during freezing storage, various types of lipoprotein particles in the plasma and granule fractions interact, resulting in gelation. Yolk recombined systems made with different proportions of plasma and granules were studied, and gel properties of these systems and compositions of their fractionated components were analyzed to test our hypothesis.

## **Materials and Methods**

### **Materials**

Fresh large Grade AA white shell eggs were obtained from farms in Ames, IA. Eggs were produced by Hy-Line W-36 laying hens raised in conventional cage housing, and hens' age was 30-35 weeks. Eggs were stored in 4°C refrigerator at the research laboratory for no longer than 7 days.

Yolks were separated following the method by Powrie et al. <sup>2</sup> with modifications. Fresh eggs were manually broken and the yolks were carefully separated from the albumen, with the chalazae removed. Each yolk with intact vitelline membrane was rolled on a paper towel to remove any remaining albumen and chalazae adhering to the vitelline membrane. The vitelline membrane was pierced to collect the pure egg yolk in a beaker. Following the harvest of approximately 1 L of pure egg yolk solution, the yolks in the beaker were slowly stirred for sample homogeneity.

### **Preparation of plasma and granules for recombined yolk systems**

Yolk was fractionated into plasma and granules using a modified method by McBee and Cotterill <sup>14</sup>. Yolk was diluted 1:1 (v/v) in deionized water and stirred until they were well-mixed. This dispersion was then centrifuged at 10,000 g for 45 minutes at 4°C, and the plasma (supernatant) was separated from the granules (pellet). The plasma was centrifuged again using the same parameter for more complete separation of plasma and granules. Following the addition of 200 ppm sodium azide for preservation, the collected fractions were stored in capped containers at 4°C refrigerator until further processing.

### **Plasma ultrafiltration to remove water**

To remove the added water from centrifugation step, the plasma solution was filtered using the Minimate™ TFF System (Pall Corporation, Port Washington, NY) with a Minimate™ Tangential Flow Filtration Capsules of 5 kD pore size. The filtration was run continuously in 4°C walk-in refrigerator until plasma volume was reduced by approximately 50%. To ensure adequate water removal, moisture content of plasma was determined using oven drying at 110°C overnight.

### **Preparation of recombined yolk systems**

Four yolk systems that would mimic (1) whole egg yolk (78% plasma, 22% granules, db), (2) pure plasma fraction (100% plasma, db), (3) plasma mixed with 50% granule fraction (88% plasma, 12% granules, db), and (4) granule mixed with 50% plasma fraction (64% plasma, 36% granules, db) were prepared by adding the filtered plasma fraction to the granule fraction using calculated proportions (Table 2.1). Moisture and total solid contents were kept constant across the four systems, 53% and 47%, respectively. Mixtures were stirred manually using



spatula before mixed with Ultra-Turrax® T rotor stator homogenizer (Laboratory Supply Network, Inc., Atkinson, NH) at 8,000 RPM for 30 seconds for a more homogenous mixture.

### **Yolk freezing and thawing for gel formation**

The yolk systems were divided into three batches: fresh, frozen, and frozen for rheology and gel measurements. For the frozen samples, 40 g of each yolk mixture was poured into a 50 mL conical polypropylene centrifuge tube, and they were vacuum-sealed in a vacuum bag with a FoodSaver® V222 vacuum sealing system (SunBeam Products, Inc., Jarden Consumer Solutions, Boca Raton, FL) to reduce freezer burn. The vacuum-sealed bag of four centrifuge tubes containing the different systems was submerged in the reservoir of a Haake SC 100 refrigerated/heated bath circulator (Thermo Fisher Scientific, Waltham, MA) filled with 1:1 ethylene glycol:Milli-Q water at 0°C. The bath was then set to -20°C. After the samples reached -20°C at a cooling rate of 0.3°C/min, they were held in the -20°C bath for 3 hours before storing in a -20°C freezer for 5 weeks.

### **Preparation of samples for rheological analysis**

Samples for rheology were prepared following a method by Au et al.<sup>13</sup> using a custom-made aluminum apparatus composed of two heat transfer blocks (23.2 cm length, 7.2 cm width, 2.5 cm height) and an aluminum mold plate (23.2 cm length, 7.2 cm width, 3 mm height) with five circles of 35 mm-diameter cutouts. The heat transfer blocks were connected with plastic tubing positioned level to the inlet and outlet ports on Haake SC 100 refrigerated/heated water bath circulator (Thermo Fisher Scientific, Waltham, MA) with 1:1 ethylene glycol:Milli-Q water mixture circulated through the blocks. When temperature reached 0°C, a sheet of Parafilm was placed over the bottom block, followed by the aluminum mold plate filled with egg yolk and

another sheet of Parafilm to cover the mold plate. The top heat transfer block was quickly placed over the filled mold plate, and the cooling bath was set to  $-20^{\circ}\text{C}$ . After reaching  $-20^{\circ}\text{C}$  at a cooling rate of  $0.3^{\circ}\text{C}/\text{min}$ , the yolks were held in the apparatus for another hour before the mold plate was removed and sealed in a vacuum bag to reduce freezer burn. The samples were then stored in a  $-20^{\circ}\text{C}$  freezer for 5 weeks.

### **Fractionation of egg yolk into plasma, granule, LDL and livetin fractions**

The four fresh and frozen-thawed yolk systems were fractionated using a method modified from Ulrichs and Ternes<sup>15</sup> and McBee and Cotterill<sup>14</sup>, immediately for fresh yolk after they were recombined and five weeks for the frozen-thawed samples. Mixtures of 1:3 yolk:deionized water were prepared and stirred until well mixed. The mixtures were again mixed with Rotor-Stator at 8,000 RPM for 30 seconds to improve homogenization. Then, 30 mL of each yolk:water mixture was aliquoted to three 50 mL conical polypropylene centrifuge tubes. The tubes were transferred to a FIBERLite F15-8x-50cy fixed angle rotor in a Sorvall Legend XT centrifuge (Thermo Fisher Scientific, Germany) and centrifuged at 15,500 g and  $4^{\circ}\text{C}$  for 1 hour. The plasma (supernatant), granule (pellet), and LDL (floating lipidic layer from frozen systems) were collected and the mass was recorded.

The collected plasma was further fractionated into LDL and livetin using a method modified from Ulrichs and Ternes<sup>15</sup>. Mixtures of 3:2 plasma:1% (w/v) carboxymethyl cellulose solution were centrifuged with the same parameter as above. The LDL (floating lipidic layer) and livetin (watery fraction) were collected and the mass was recorded.

**Determination of mass balance of all fractionated yolk components**

Small amounts of all fractions were taken for moisture/solid content analysis. Samples were 130°C oven-dried overnight, transferred to a desiccator for 5 minutes, and measured for change in mass. Moisture and solid contents were calculated to allow all data to be converted to dry mass balance (db). Fraction distributions (%) within each system were then compared among yolk systems and between fresh and gelled yolk for changes in fractionation behavior caused by freezing and thawing.

**Protein characterization by gel electrophoresis**

Protein distributions of fresh and frozen samples of all four recombined yolk systems were studied using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) performed according to Bio-Rad instructions and a modified method from Laca, Paredes, and Díaz <sup>16</sup>. Samples of different fractions from each system were diluted in deionized water based on their estimated protein content quantified using bicinchoninic acid (BCA) assay and then 1:1 (v/v) in a mixture of 95% Bio-Rad 2x Laemmli Sample Buffer (Tris-HCl/glycerol/bromophenol blue) and 5%  $\beta$ -mercaptoethanol, to bring sample concentrations to approximately 1  $\mu$ g protein/ $\mu$ L. After dilutions, samples were heated in boiling water bath for 6 minutes. Protein standard Precision Plus Protein™ Dual Color Standards (BioRad Laboratories, Inc., Hercules, CA) and samples containing fractions from the same system were loaded onto a Bio-Rad Mini-PROTEAN® TGX™ precast polyacrylamide gel (4–20% gel, 12-well, 20  $\mu$ L) at volume of 5  $\mu$ L for standard and 7  $\mu$ L for samples, and electrophoresed at 175 V for 35 minutes using the standard SDS-PAGE running buffer (250 mmol Tris, 1.92 mol glycine, and 10 g SDS per L).

The gels were fixed in a solution consisting of 40% methanol, 10% acetic acid and 50% deionized water for 30 minutes. After removing the solution, 50 mL Bio-Safe™ Coomassie F-250 Stain was added to stain the gels, and gels were gently shaken for 1 hour before rinsed with deionized water for 30 minutes. Gels were scanned with an ImageScanner flatbed scanner (Amersham Pharmacia Biotech Inc., Piscataway, NJ) for quantification and kept in water for storage.

For densitometry analysis, the scanned images of the gels were processed with Image Processing and Analysis in Java software, ImageJ (National Institutes of Health, Bethesda, MD). An external standard was used for calibration following the NIH optical density calibration procedure <sup>17</sup>. Each lane on the gel was plotted as a density spectrum where each peak represented a protein band. Each protein band was compared to published literature <sup>18</sup> for protein identification and the optical density, or peak area, was determined. Density is reported in percent relative optical density units (%OD).

### **Rheological analysis**

After five weeks of freezing, yolk discs were analyzed using an Ares-G2 rheometer (TA Instruments, New Castle, DE) with a set of 25 mm diameter parallel plates. Sample thawing was performed for one disk at a time. A polyvinyl chloride cylindrical plunger (35 mm diameter, 62 mm height) was used to push the yolk disk out of the mold to thaw on the bottom parallel plate at room temperature (23°C) for 15 minutes.

All four yolk systems, 10 replicate discs each, were subjected to an oscillation amplitude sweep test. Normal force of 0.2 N was applied for all samples. Oscillation strains in the range of 0.1-10% was applied at a frequency of 1 Hz with 41 steps (data points). The average elastic

modulus ( $G'$ ) within the linear viscoelastic region (LVR) was reported as a measure of gel strength. Yield stress ( $\sigma^*$ ) was obtained as the stress where a 10% reduction from the average  $G'$  of was achieved.

With outliers removed, average  $G'$  and  $\sigma^*$  were analyzed for significant difference. Values outside  $\text{mean} \pm 2\text{SD}$  range were considered as outliers. With the outliers eliminated, 9 discs from system 1:0, 5 discs from system 1:0.5, 8 discs from system 1:1, and 9 discs from system 1:2 were used for data analysis.

### **Statistical analysis**

Statistical analysis was performed for rheology samples (1 treatment replication, >5 samples replications) with JMP Pro 12, statistical software from Statistical Analysis System (SAS) Institute Inc. (Cary, NC). One-way analysis of variance (ANOVA) tests were conducted, and significance of difference ( $p\text{-value} < 0.05$ ) was calculated using Tukey's HSD (honest significant difference) test.

## **Results and Discussion**

### **Rheological analysis of the various yolk systems**

Strain sweep test with oscillating force allows combined measurement of viscosity and gel behavior (viscoelasticity) of the gelled yolk systems. The  $G'$ , or elastic modulus, represents the solid-like behavior of a material as it goes through increasing levels of stress and strain. Within the linear viscoelastic region (LVR), in which  $G'$  is constant, the material macrostructure has not been deformed<sup>13</sup>. As shown on Figure 2.2A,  $G'$  increased significantly ( $p < 0.05$ ) with the increasing amount of granules in the system. This finding shows that both plasma and granules

contribute to gel formation and strength in frozen-thawed egg yolk, and aggregations involving larger quantity of granules with other plasma components might have enhanced the gel stability due to the higher proportion of the larger granule size. Au et al.<sup>13</sup> found an increase in particle size with extended freezing storage, indicating that yolk remained a dynamic system even at -20°C and that aggregations of lipoproteins continue as the states of water changes. In gelled yolk, the amount of granules was found to have direct influence on the force required to change the solid-like behavior of the gel into a viscous liquid<sup>13</sup>.

Yield stress ( $\sigma^*$ ) is the minimum amount of stress needed to induce flow in a structured fluid. The flow will occur when the stress is sufficient to disrupt the material gel structure. Yield stress determines many aspects of a material processing, handling, storage and performance properties. In this case, the  $\sigma^*$  could be used as an indicator of ease of mixing, in which higher  $\sigma^*$  corresponds to greater difficulty of mixing. Figure 2.2B shows no significant difference in  $\sigma^*$  between yolk systems, except for system 1:0.5 which could be an experimental error due to substantially lower number of replications and a relatively high standard deviation. These results suggest that this gel characteristic is not significantly affected by proportions of plasma and granule.

Figure 2.3 shows how  $G'$  and yield stress are affected by granule amount, and if there is any linear correlation between these factors. Gel strength is more sensitive to changes compared to yield stress. There is a strong positive linear correlation between the amount of granule in the systems and gel strength. Chang et al.<sup>5</sup> also found similar trends where viscosity of gelled yolk increased with increasing granule content. Particle size and quantity have direct influence on material properties, such as viscosity and texture<sup>19</sup>. Granules consist of circular complexes ranging in diameter from 0.3 to 2  $\mu\text{m}$ <sup>20</sup>. The diameter of LDL spherical nanoparticle ranges from

17 to 60 nm<sup>21</sup>. The incorporation of higher amount of larger particles, i.e. granules, into the system is likely to increase viscosity, but they have to be participating in gel formation with other proteins to increase gel strength, which was measured as  $G'$ . While gels become significantly harder with more granules in the systems, the amount of force required to deform the yolk was relatively similar. It is proposed that granules participate in gelation by forming aggregates with other plasma components, resulting in larger aggregates than those formed by plasma components alone, leading to significantly higher gel strength as measured through  $G'$ . However, the chemical bond or nature of interactions formed with or without granules are the same, thus  $\sigma^*$  remained unchanged.

### **Analysis of mass balance of all fractionated yolk components**

Fractions obtained from yolk system fractionations include plasma, granule, floating LDL (only in gelled yolk), and LDL and livetin from further fractionation of plasma as shown in Figure 2.4. Egg yolk was separated to plasma and granule fractions based on density difference after the addition of water and centrifugation. In frozen systems, this fractionation also resulted in a third phase which appeared as a floating lipidic paste, and it is referred to in this study as floating LDL. Plasma was diluted with 1% CMC solution, and fractionation results in upper LDL fraction and water-soluble livetin fraction<sup>22</sup>. CMC can cause an agglomeration of proteins, including whey protein, soy protein, caseinate and bovine serum albumin<sup>23, 24</sup>. Imeson et al.<sup>23</sup> described that the polysaccharide-protein interactions are primarily electrostatic in nature and increase as the net positive charge on the protein increases; therefore the interactions are very sensitive to changes in ionic strength and pH. In egg yolk plasma, agglomeration of LDL

particles is caused by electrostatic interaction between the negatively charged carboxyl group of the CMC and the positively-charged side chains of the amino acids in the LDL proteins <sup>15</sup>.

The mass distribution data in Figure 2.4 allowed comparisons between fresh and frozen-thawed yolk within the same system, and also between systems. Three main trends were observed. Firstly, freezing promoted LDL aggregation, which could be evidenced by the appearance of a third floating phase in the yolk during plasma and granule fractionation in all four frozen systems. Through SDS-PAGE, this third layer was confirmed to be LDL as they have similar protein compositions as the LDL fraction obtained by the addition of CMC. This will be discussed more in depth in the following sections of this work. LDLs are water-soluble spherical nanoparticles with a lipid core of triglycerides and cholesterol ester surrounded by a monofilm of phospholipid and proteins <sup>25</sup>. Larger aggregations formed between the LDL allowed this complex to float when centrifugal force was applied due to the LDL's relatively low density, 0.982 g/mL <sup>25</sup>. Results showed that in the absence of granule, yolk is more prone to LDL aggregation during freezing. In system 1:0, more than half the mass of the frozen system was separated into the floating LDL fraction, very few proteins remained in the water-soluble plasma fraction. However, the amount of floating LDL was significantly reduced with the presence of granule in the system. System 1:0.5 only had 25% floating LDL in comparison to 53% in the 1:0 system, although there was only 12% reduction in the amount of plasma in the system.

Secondly, LDL-HDL-livetin interactions also occurred during freezing, in addition to LDL-LDL interactions, which is the most reasonable explanation to explain the above observation. Except for the system 1:0, all other systems showed increasing amount of granule fraction obtained after freezing, especially in system 1:0.5 where granule content doubled in amount in the frozen system relative to the fresh system. Compared to the first two systems, the



systems with high granule to plasma ratio contained more balanced proportions of LDL and livetin (eg. 3:5 and 3:4 LDL:livetin ratios in 1:1 and 1:2 yolk systems, respectively). The substantially high mass in the granule fractions indicates that aggregations have involved other components other than LDL. HDL-LDL aggregations could have occurred. Also, the disruption of granule assembly resulting from concentration of ions following ice crystal formation might have occurred <sup>5</sup>. The liberated granule components were available to form complexes with the plasma LDL and livetin proteins. Chang et al. <sup>5</sup> found through electrophoretic and optical density studies that the migration of LDL bands following gelation were restricted and that some livetin bands were not visible, suggesting that LDL micelles and livetin might have aggregated during frozen storage. During centrifugation, these complexes might have separated with the granule fraction due to the higher density.

Thirdly, it was observed that the proportions of fractions obtained did not fully reflect the amount used in the systems. For the fresh systems, the mass distribution of plasma and granule does not represent the amount added in the system, especially for system 1:1 and 1:2. Instead of having 78% plasma and 22% granule, the fractionation of 1:1 yolk resulted in 45% plasma and 55% granule. Similarly, system 1:2 fractionation resulted in 33% plasma and 67% granule, instead of 64% plasma and 36% granule. The entrapment of plasma protein in granule might have occurred during the initial plasma and granule separation. The purity of the fractions obtained through the used method was not measured, but it was speculated that 1:1 dilution with water might not have been sufficient to completely separate the plasma from the granule fraction. Sedimentation velocity plays an important role in controlling the sedimentation behavior of particles in liquid phase. Increased solid contents increases hindering effects like counter flow of displaced liquid or rise in density and viscosity of the suspension. However, according to

Strixner and Kulozik <sup>26</sup>, a dry matter reduction below 29% combined with g-forces up to 10,000g should result in excellent separation efficiencies of the plasma and granule fractions.

These unexpected findings suggest that the recombined yolk systems do not fully resemble natural yolk. Compared to previous work by Au et al. <sup>13</sup> where egg yolks formed relatively strong gels within a few days of freezing, these systems required at least a 3 week freezing period to reach similar levels of gel strength. Gels with shorter freezing time were not fully formed and did not give valid rheology results. Besides slightly higher moisture content, the change in yolk characteristic could have been caused by changes in the yolk physiological condition during the ultrafiltration process, and not because of the mild physical treatment (eg. homogenization using rotor-stator). In a preliminary study where homogenization was applied to normal yolk, no change in fractionation behavior was observed. A study by Sirvente et al. <sup>27</sup> confirmed no change in protein solubility, apparent viscosity, and particle size in yolk and plasma homogenized with rotor-stator at 20,000 RPM for 1.5 min. Therefore, no damage should have been caused during homogenization at 8,000 RPM for 30 seconds. Along with water, other salts and minerals or any other molecules with molecular weights lower than 5 kDa might have been filtered out of the plasma during the concentration step causing a change in yolk lipoprotein's separation behavior.

Previous studies explained that the microstructure of egg yolk, particularly granules, closely depends on pH, ionic strength and the presence of bio- or polyvalent mineral cations <sup>28</sup>. In other study, it was described that granules were disrupted due to the addition of concentrated salt solution. Upon dialysis, elimination of Na<sup>+</sup>, Cl<sup>-</sup> and Ca<sup>2+</sup> ions occurred. The demineralized granule LDL and HDL had a zero net charge and were precipitated <sup>29</sup>. Similarly, some plasma LDLs used in this study might have been destabilized through the removal of minerals during the

plasma concentration. Some oil mass was found attached to the wall of the centrifuge tubes after the plasma-granule fractionation of the fresh systems. This suggested that LDL micelle might have been partially broken/destabilized, causing the lipid to break out and protein to precipitate <sup>9</sup>. This explains why the centrifugation resulted in very high amount of granules. In this study, all comparisons were made only among the recombined systems treated under the same conditions. Thus, interpretation of changes in the mass balance distribution between systems is still valid.

Despite of the variations of the plasma and granule proportions, the LDL and livetin proportions remained similar across the fresh systems, which was approximately 1:1 ratio. The LDL and livetin together represent the amount of plasma protein in the system; however, deviations were noticed when compared to literature where plasma should have been composed of 85% LDLs and 15% livetins. According to Ulrichs and Ternes <sup>15</sup>, the separation of LDL and livetin fractions using CMC allowed for fraction purity of about 88%, meaning that some LDLs might not be completely separated from the livetin fraction. Another possible reason was that the LDL was precipitated with the granule fraction as mentioned previously. The latter explanation is more likely since there was a significant increase in granule mass balance, and not in the livetin fraction.

Overall, this mass balance distribution analysis provides insight of what might have happened during gelation. In system 1:0 and 1:0.5, where granule content is less than the natural content in natural yolk, plasma fractionation after gelation resulted in substantially high amount of floating LDL fraction, reflecting extreme LDL aggregation during gelation. In the other systems as well as the first two fresh yolk systems, both fresh and gelled yolk's plasma contained balanced proportion of LDL and livetin. This portion might represent the not yet/lightly aggregated LDL particles and livetin, where separation of major part of LDL has to be performed

with the addition of CMC. The increased granule contents in all systems also indicated the interaction between HDL, LDL and other proteins. We hypothesized that the presence of granule helps to stabilize the yolk network by directly aggregating with LDL. Also, freezing causes ice crystal to form, leading to the concentration of ions and yolk components. Under high ion concentration, granules are disrupted and the liberated granule components including LDL and HDL are available to interact with the plasma components. By centrifugation, these aggregates are precipitated due to the higher density, explaining the increase in the mass of the granule fraction after gelation.

### **Analysis of protein distribution in fractions obtained from fractionated yolk systems**

A total of 4 SDS-PAGE gels (one gel for each system) containing the recombined egg yolk, plasma, granule, LDL and livetin fractions obtained from both fresh and gelled yolks were analyzed. Gel electrophoresis analysis of the fractions from the different systems allowed better understanding of which proteins participated in freeze-thaw gelation by observing changes in protein band. The protein bands in each lane represented all protein subunits contained in each fraction. Based on previous literature<sup>18</sup>, the obtained bands with expected molecular weight (MW) were identified. Bands of molecular weight 33 and 36 kDa are  $\beta$ -livetin, 55 and 73 kDa are  $\alpha$ -livetin and 203 kDa is  $\gamma$ -livetin. Bands of 15, 17, 55, 68, 85, 93, 122 and 221 kDa represent apo-LDL, and 31, 47, 78, and 110 kDa are apo-HDL. Two bands located between band 93 and 110 kDa were not identified in previous literatures, but they are believed to be apo-HDL since they are most predominant in the granule fraction. All bands were quantified and reported in relative optical density (%OD) to allow quantitative comparisons between samples.

Densitometry analysis of the gels confirmed that the separated plasma and granule fractions, as

well as the further fractionated fractions were far from pure. In unfrozen samples, granular proteins (apo-HDL) were found in the plasma fraction and plasma proteins (most apo-LDL) were found in the granule fraction. This could be due to what was previously discussed on the change in yolk physiological conditions during processing that affected separation behavior. Therefore, comparisons between before and after gelation should only be made within the same recombined systems.

Relative OD analysis as shown in Table A.1 indicates a decrease in HDL proteins with MW 31, 47 and 110 kDa in the granule fraction after gelation. In contrast, higher OD of these proteins, especially the 110 kDa protein, were found in the LDL fractions obtained from gelled yolk. Similar trends were observed in all the systems (Figure 2.5). Au et al.<sup>13</sup>, in the study focusing on yolk gelation in relation to freezing storage time, also found similar results. Apo-HDLs of 31 and 110 kDa were found to be in significantly higher OD in gelled plasma than fresh plasma. However, they did not separate floating LDL from the plasma, or further fractionated plasma into LDL and livetin, which explains why they found a change in the plasma fraction and not in the LDL fractions. The large amount of granule component HDL in the LDL fractions after gelation indicates a breakage of granule assembly and release of its component. Also, a strong LDL-HDL interaction was formed, which is likely to be a hydrophobic interaction as suggested by Au et al.<sup>13</sup> because their Native-PAGE did not show a higher molecular weight protein band in the gelled plasma fraction.

LDL proteins with molecular weights of 11, 17, 93 and 122 kDa, which are normally present in the livetin fraction, were completely absent from the gelled livetin fraction, as shown on the two lanes on the left part of each gels on Figure 2.5. Tracking backwards, these LDL proteins were also shown to have lower OD in gelled plasma (Table A.3). We hypothesize that

these very high and very low MW proteins are preferentially involved in aggregations and might have partitioned into the granule (11 and 17 kDa) or LDL fractions after gelation (Table A.2, A.5). Although the difference in OD appeared to be subtle, it is important to take into account the mass distribution, where gelation resulted in higher amount of LDL and granule fractions. In addition, the protein profiles of both LDL and floating LDL fractions after gelation did not show any marked differences, which suggest that aggregations were purely physical and the larger aggregated complexes floated more easily during centrifugation.

Livetin proteins including  $\alpha$ - and  $\beta$ -livetins were found to have different trends, as described on Table A.3.  $\alpha$ -Livetin, which include 55 and 73 kDa proteins were found to decrease in the livetin fraction after gelation. Conversely, these proteins were found to increase in the granule fraction after gelation.  $\beta$ -Livetin, which include proteins 33 and 36 kDa, were observed in higher proportions in livetin after gelation. This suggested that the higher molecular weight livetin could be involved in aggregation with the LDL-HDL proteins, explaining why it was separated along with the granule fraction. The lower molecular weight livetin remain soluble in the watery fraction, and the OD increase could have been due to the missing LDLs from the livetin fraction, making livetin the predominant proteins in the fraction.

### **Proposed yolk frozen-thawed gelation mechanism supported by experimental evidence**

Many past studies have confirmed that gelation is caused by aggregation of LDL proteins<sup>2, 8-10, 30, 31</sup>, but some also suggested that gelation may involve compounds other than LDL<sup>5, 7, 13, 32</sup>. This study suggested that as LDL aggregated, some high molecular weight livetin proteins and HDL from the disrupted granules also form interaction with the LDL aggregates, resulting in a heterogeneous aggregation consisting of different proteins of various molecular weights.

Fraction separations through centrifugation approved this hypothesis through the appearance of a third floating LDL layer and significant increase of mass in the granule fraction. For future work, methods modification might be needed to obtain very pure plasma and granules with their natural physiological conditions and properties retained when constructing yolk systems to study protein and mass distribution.

A possible mechanism for egg yolk gelation is proposed in Figure 2.6. Gelation involves the aggregation of lipoprotein and livetin proteins from both plasma and granules initiated by water crystal formation during slow freezing. Furthermore, disruption and liberation of lipoprotein particles from granules can lead to aggregation between the hydrophobic regions of the proteins, which previously might be inaccessible. Disruption of granules released both LDL and HDL particles, and these proteins became available to interact with plasma LDL and form aggregates. Without granule in the yolk system, the LDL proteins, especially the ones with low and high molecular weights are more prone to aggregation. The more rigid granule structure can also provide a stabilizing force in the gel network. Pure LDL-LDL aggregations were interrupted as HDL and livetin participated. Most studies that support that granules are involved in gelation only focused on the LDL component of the granule forming interaction with the plasma LDL. However, our results show that yolk protein aggregation involves more than just LDL components, but also HDL and livetin.

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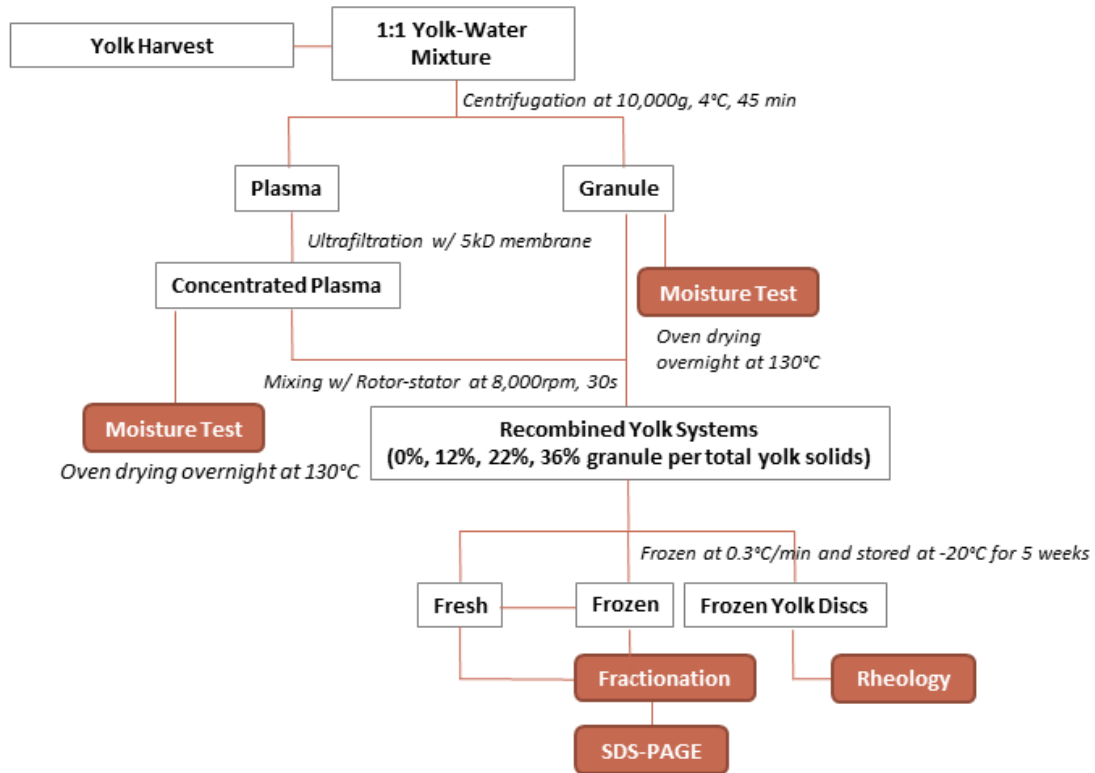


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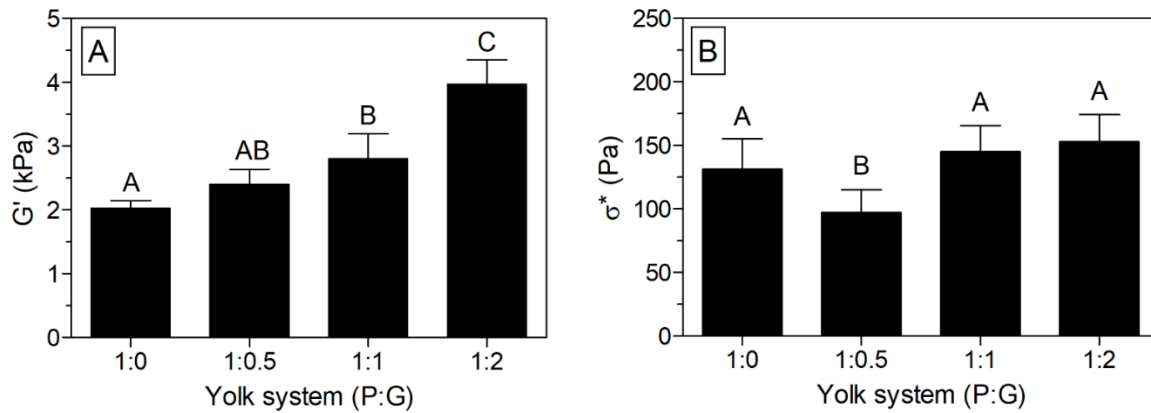
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**Table 2.1.** Yolk system ID based on plasma (78%) and granule (22%) native composition in hen egg yolk

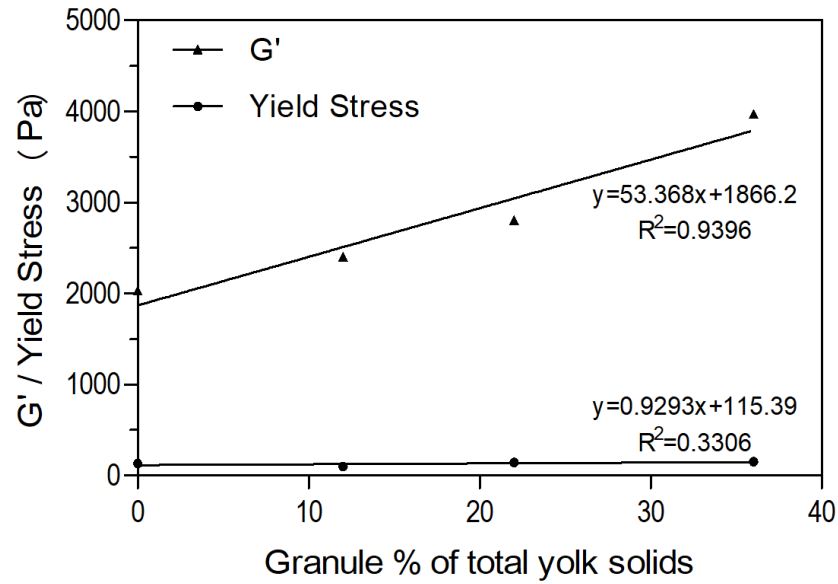
Yolk Systems	Natural Yolk P:G Ratio	P:G Ratio (Dry Weight Basis)	% Granule
Plasma	1:0	78:0	0
Plasma + 50% Granules	1:0.5	78:11	12
Whole Yolk	1:1	78:22	22
Granules + 50% Plasma	1:2	78:44	36



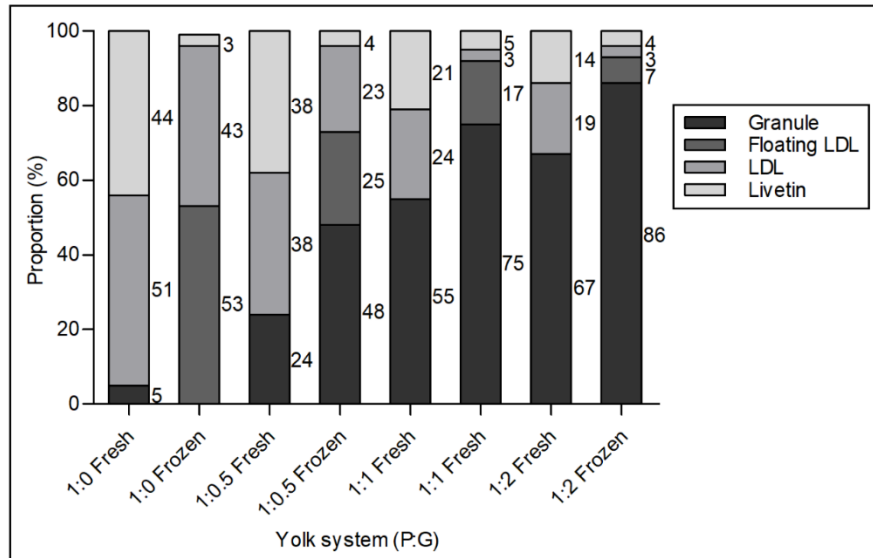
**Figure 2.1.** Flowchart of recombined egg yolk system preparation and treatments.



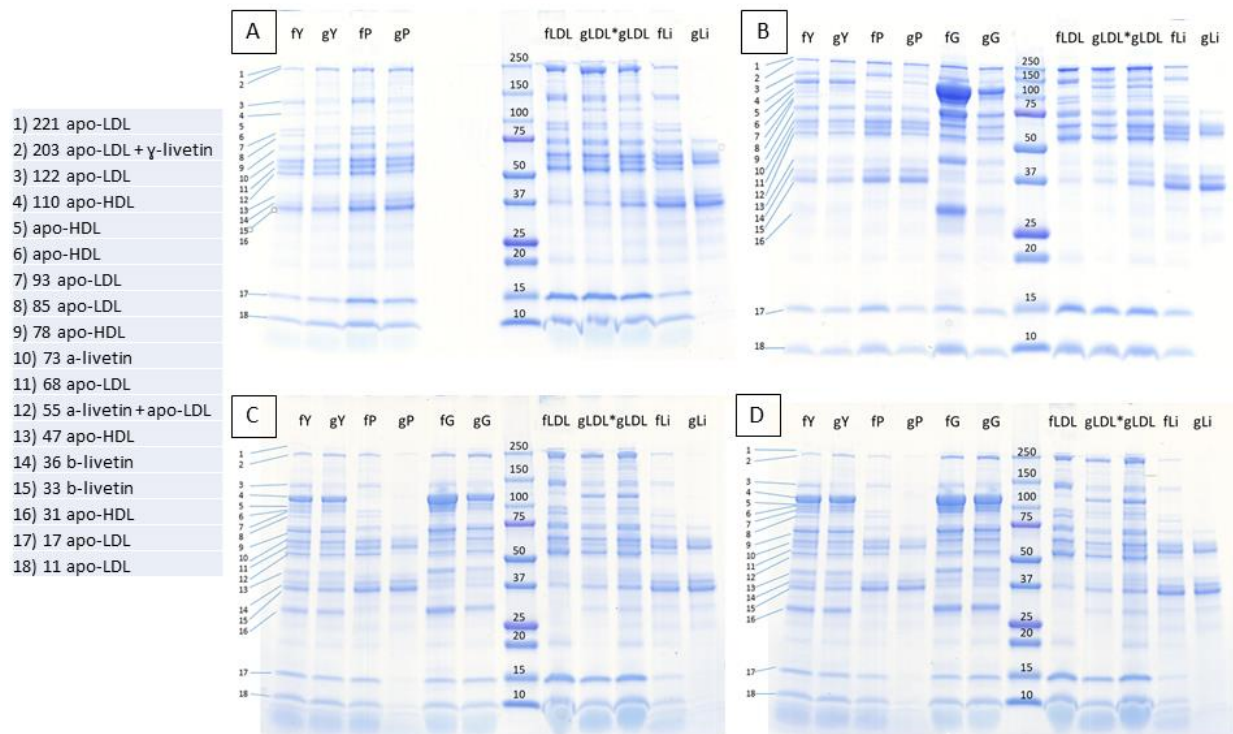
**Figure 2.2.** Elastic modulus ( $G'$ , A) and yield stress ( $\sigma^*$ , B) of gelled recombined yolk systems stored at -20 °C for 5 weeks and thawed. Values with different letters are significantly different ( $p < 0.05$ ).



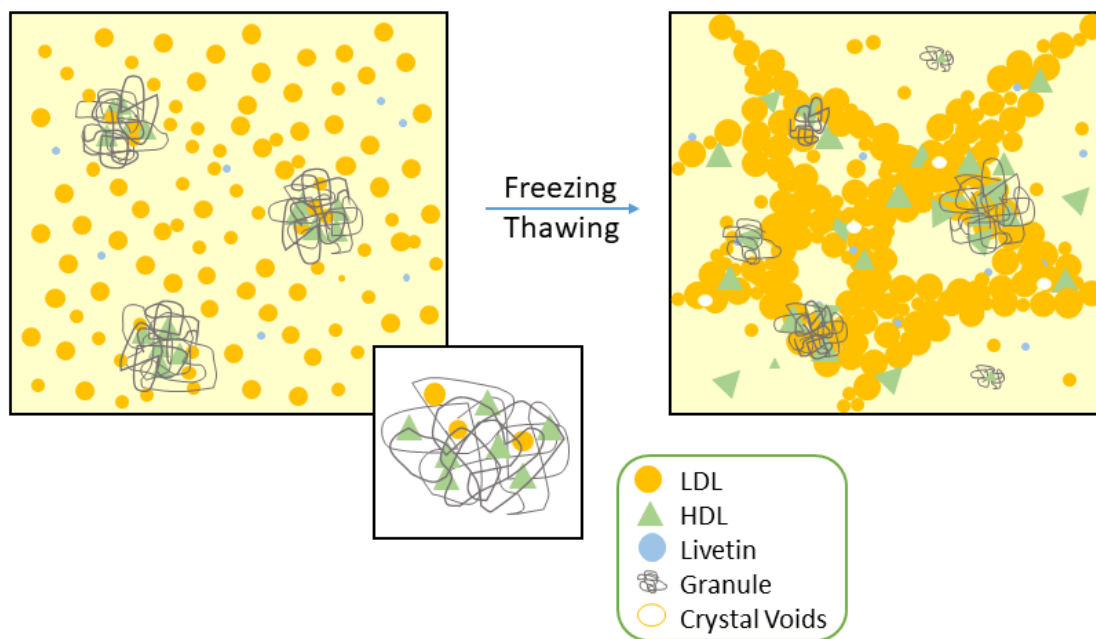
**Figure 2.3.** Effect of granule content in yolk on gel strength.



**Figure 2.4.** Dry mass balance distribution of yolk fractions in recombined egg yolk systems before and after freezing and thawing.



**Figure 2.5.** SDS-PAGE of yolk, plasma, LDL and livetin of yolk system A-D (A, 1:0; B, 1:0.5; C, 1:1; D, 1:2). Proteins were identified based on SDS-PAGE profiles by Guilmineau et al. Abbreviations are Std, standard; f, fresh; g, gelled; Y, yolk; P, plasma; LDL, LDL; Li, livetin; \*gLDL, floating LDL from plasma-granule fractionation.



**Figure 2.6.** Proposed mechanism of protein distribution in yolk before and after freezing and thawing.

### CHAPTER 3. DETERMINATION OF FOOD ADDITIVES AND TREATMENTS TO PREVENT GELATION IN FROZEN-THAWED EGG YOLK

A manuscript to be submitted to *Food Chemistry*

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#### Abstract

This study demonstrated advances in preventing egg yolk gelation during freezing and thawing. Gelation negatively affects yolk functionality in food processing, and preventing gelation using 10% salt or sugar limits the application of the yolk due to significant change in flavor. Several food additives other than salt and sugar were tested as attempts to prevent gelation during freezing and thawing. Significant reduction ( $p < 0.05$ ) in hardness of frozen-thawed yolk (45 hours freezing at  $-20^{\circ}\text{C}$ , 4 hours thawing at  $25^{\circ}\text{C}$ ) indicates that hydrolyzed carboxymethyl cellulose (HCCM), proline, and egg peptides from hydrolyzed yolk and white are effective in inhibiting gelation. The mechanisms in which these additives prevented gelation were further studied through measuring the changes in the amount of freezable water, protein size, and protein surface hydrophobicity. Overall, this study provides insight to several gelation inhibitors that may replace the use of salt or sugar in commercial frozen egg yolk.

#### Introduction

Egg yolk, in its fluid form, is a valuable food ingredient for the manufacture of many food products. Large amounts of liquid yolk are frozen commercially for prolonged storage of up to 1 year (Rembrandt, 2017). The benefits of storing egg yolk in the frozen state are prevention

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of microbial growth and spoilage, retention of egg yolk flavor and color, and inhibition of chemical reactions such as autoxidation of lipids and the browning reaction (Powrie, 1968). However, when yolk is frozen and stored below  $-6^{\circ}\text{C}$ , an irreversible alteration in fluidity known as gelation occurs (Moran, 1925). This physiological change is undesirable because of reduced yolk dispersibility in water and functionality.

The mechanism for yolk gelation caused by freezing and thawing has not been fully elucidated. Regardless of the many existing proposed mechanisms, most researchers agree that ice crystal formation during freezing storage plays a fundamental role in yolk gelation. Moran (1925) found that when yolk is rapidly cooled below  $-6^{\circ}\text{C}$ , no significant viscosity change could be seen. Lopez, Fellers and Powrie (1954) and Jaax and Travnicek (1968) found that when yolk was frozen rapidly in liquid nitrogen ( $-196^{\circ}\text{C}$ ) and stored at about  $-20^{\circ}\text{C}$  for periods up to 49 days, the apparent viscosities of the thawed products were lower than those of the controls frozen and stored at approximately  $-20^{\circ}\text{C}$ . Additionally, Rolfe (1969) stated that ice crystal formation needs to reach an extent of 81% in order for gelation to occur.

Other than rapid freezing, some other treatments have been applied in frozen yolks to prevent gelation. Inhibition of gelation could be achieved by the addition of cryoprotective agents, proteolytic enzymes, or mechanical treatments to prevent ice crystal formation and changes in the yolk physicochemical conditions that favor aggregation of proteins. Moran (1925) was the first to report that a food additive, sucrose, could be used to prevent gelation of yolk. Other additives such as glucose, arabinose, galactose, glycerol, sorbitol, propylene glycol and salt (NaCl) have also been found to be effective inhibitors of gelation (Lesser, 1948; Lopez, Fellers, & Powrie, 1954; Powrie, Little, & Lopez, 1963; Thomas & Bailey, 1933). At low concentrations, salts were shown to stabilize the system due to electrostatic shielding of

attractive forces (Hamada, Tanaka, Tartaglia, Pawar, Vendruscolo, Kawamura, et al., 2009). Crotoxin (lecithinase A) at 1mg/mL yolk and 10 mg/mL yolk used led to only 10-20% gelation compared to untreated yolk (Feeney, MacDonnell, & Fraenkel-Conrat, 1954). The LDL and HDL fractions were proposed to be attacked by the enzyme and the resultant lysophospholipoproteins had an altered solubility in water. Papain at 0.05% concentration was also reported to inhibit gelation due to its ability to break down the proteins responsible for gelation (Lopez, Fellers, & Powrie, 1955).

Increased consumer awareness towards healthy consumption of food low in salt and sugar has been our motivation to reexamine this issue. With more advanced technology and new research effort on applications of food additives to improve functionality, we plan to find alternative methods to inhibit gelation, without significantly altering the yolk flavor. Physical means, such as colloid milling will be introduced to destroy or “bury” the native yolk granular and plasma LDL surface structures responsible for gelation, and combinations of food additives such as hydrolyzed carboxymethyl cellulose (HCMC), hydrolyzed egg white (HEW), hydrolyzed egg yolk (HEY), proline, polyethylene glycol and tween 80 will be evaluated for their effectiveness in interrupting protein association thus inhibiting gelation. These additives were selected due to their high solubility in water, low freezing point, and/or presence of a hydrophobic side chain.

The specific impact of additives on protein interactions can vary greatly and is usually dependent on the chemical nature, additive concentration, protein type, protein concentration and pH. In this study, we systematically tested the effectiveness of each additive at varying concentrations as well as mechanical treatments such high speed mixing and colloid milling on yolk gelation reduction. Synergistic effect of combined treatments was also explored. With the

selected additives, the mechanism of gelation prevention was further studied. We hypothesized that since gelation may be associated with ice crystal formation which then lead to dehydration and aggregation of lipoproteins, treatments that can reduce the amount of freezable water, minimize exposure of hydrophobic site, and/or prevent surface aggregation can prevent gelation. To prove our hypothesis, the amount of freezable water, protein surface hydrophobicity, and particle size in yolk before and after freezing were evaluated and compared.

## **Materials and Methods**

### **Materials**

Fresh Grade A white shell eggs were obtained from grocery stores in Ames, IA. Eggs were stored in 4°C refrigerator at the research laboratory. Hydrolyzed carboxymethyl cellulose (HCMC), hydrolyzed egg white protein (HEWP), hydrolyzed egg yolk protein (HEYP) were prepared with the methods described in the later sections. Arginine, proline, Tween 80, polyethylene glycol 200 (PEG 200), and other chemicals were purchased from Fisher Scientific (Hampton, NH).

### **Preparation of hydrolyzed carboxymethyl cellulose (HCMC)**

HCMC was prepared following the optimal conditions found by Sreenath (1993). A 4% (w/w) solution of CMC in deionized water was mixed overnight. The solution was heated in an incubator to 50°C before the pH was adjusted to 4.8 with 2 M hydrochloric acid solution. Cellulase DS enzyme was added at a concentration of 1% based on the CMC substrate and the solution was mixed for 18 hours in a shaking incubator set at 45 RPM. After the reaction was completed, the solution was boiled for 30 minutes to deactivate the enzyme. The concentration of reducing ends of HCMC was measured using Somogyi-Nelson method (Kulchaiyawat, 2015).

The standard curve was established using serial dilutions of 1 mg/mL solution of glucose. The standard solutions and samples were measured at 520 nm, and the absorbance of the HCMC sample was interpolated into the standard curve to determine the concentration of free reducing ends. The average molecular weight of the HCMC was estimated to be approximately 2.9 kDa.

### **Preparation of HEWP and HEYP**

Fresh egg white and yolk were hydrolyzed using the method by Ruan, Chi and Zhang (2010). Due to the high lipid content that might interfere with the hydrolysis process, egg yolk was defatted prior to hydrolysis. Egg yolk lipids were extracted using Folch method (Folch, Lees, & Sloane-Stanley, 1957). Fresh egg yolk was mixed in 2 parts of 2:1 (v/v) chloroform-methanol solution in a shaking incubator for 30 minutes at ambient temperature. The mixture was vacuum-filtered using No.2 Whatman paper, and the filter cake was air dried for 12 hours to remove solvent.

The egg protein was dispersed in deionized water at 10 g protein (dry weight) / L water, and thermally denatured in 90°C water bath for 15 minutes. The pH of the denatured solution was adjusted to 2 using 2 M hydrochloric acid solution. The hydrolysis reaction was performed for 3 hours after adding pepsin at a selected concentration, and the temperature and pH were maintained at 45°C and 2, respectively. Inactivation of pepsin was achieved by increasing the solution pH to 7 with 2 M sodium hydroxide solution. The hydrolysates were centrifuged at 4,000 g for 15 minutes, and the supernatant was collected and lyophilized.

### **Preparation of frozen-thawed yolk samples**

Yolks were separated following the method by Powrie, Little and Lopez (1963) with modifications. Fresh hen eggs were manually broken, and the yolks were carefully separated

from the albumen, with the chalazae removed. Each yolk with intact vitelline membrane was rolled on a paper towel to remove any remaining albumen and chalazae adhering to the vitelline membrane. The vitelline membrane was pierced to collect the pure egg yolk in a beaker. The yolk was slowly stirred for homogeneity.

Additives at various concentrations (1-10% w/w) were added to yolk to make 50 g yolk mixtures and stirred with a spatula before mixed using Ultra-Turrax® T rotor-stator homogenizer (Laboratory Supply Network, Inc., Atkinson, NH) at 8,000 RPM for 90 seconds for a thorough mixing. Three replicates of 10 g yolk mixtures were distributed to three Evergreen Scientific Dilution Vials (Fisher Scientific, Hampton, NH), and were stored in a -20°C freezer for 45 hours. The freezing rate was calculated to be 0.15°C/min. The yolk mixtures were thawed for 4 hours at 25°C before analyzed for hardness.

To test the effect of mechanical treatments, fresh yolk was processed with a rotor-stator homogenizer and colloid miller prior to freezing. For the rotor-stator homogenizer, fresh yolk was processed at 8,000, 13,500 and 24,000 RPM for 90 seconds. The shear rates were calculated to be 13,299, 22,443, and 39,898 s<sup>-1</sup>, respectively. For colloid miller, two liters of fresh yolk was run through the Charlotte Colloid Mill (Chemicolloid Lab's Inc., Garden City Park, NY) at 0.003-inch clearance (shear rate: 18,618 s<sup>-1</sup>) for three passes based on the conditions found to be the most optimal in lowering yolk viscosity post gelation (Lopez, Fellers, & Powrie, 1954). The processed fresh yolk was then used to prepare freeze-thawed yolk samples, and additives were used to determine the effect of combining additive with mechanical treatments.

### **Texture analysis of frozen-thawed yolk samples**

Tests were carried out with a TA.XTPlus Texture Analyzer (Stable Micro Systems, United Kingdom) with a load cell of 50 kg. Penetration test with a penetration distance of 10 mm

and a speed of 1 mm/sec was performed using a cylindrical probe (TA-10) to characterize the frozen-thawed yolk gels of 10 g kept in 20 mL vials. The maximum force recorded corresponds to hardness, as was reported as the mean of three replicates.

### **Quantification of freezable water in selected yolk samples**

Content of freezable water was determined following the differential scanning calorimetry (DSC) method reported by Au, Acevedo, Horner and Wang (2015). DSC was performed on fresh yolk and yolk mixtures containing various additives. Exothermic and endothermic transition heats of 10-15 mg sample in aluminum hermetic pans with sealed lids were measured in four replicates. Scanning conditions were modified from Kamat, Graham, Barratt and Stubbs (1976) and Wakamatu, Sato and Saito (1983). Each sample was held for 1 min at 20°C, cooled to -50°C at 1°C/min rate and held at that temperature for 1 min, then heated from -50°C to 20°C at 10°C/min rate.

Melting temperature ( $T_m$ ) and the heat of fusion, or change in enthalpy ( $\Delta H$ ), of exothermic and endothermic peaks were obtained. The amount of freezable water in yolk was calculated following the method by Wakamatu et al. (1983). The exothermic or endothermic heat was divided by the corresponding heat of fusion of pure water (242.88 J/g for cooling and 320.62 J/g for heating). Freezable water content was reported as the average of the exothermic and endothermic freezable water values per gram solid.

### **Particle size analysis**

Particle size distributions of fresh and frozen-thawed yolk were measured by laser diffraction (LD) method using Malvern Mastersizer 2000 particle size analyzer with Hydro 2000 MU large volume wet sample dispersion unit (Malvern Instruments, Inc., Worcestershore, UK)

(Au, Acevedo, Wang, & Horner, 2015). All samples were diluted at a sample:deionized water ratio of 1:1.5 (v/v) and mixed for 1.5 hours on a stir plate until homogeneous. Diluted samples were added dropwise to a 1 L beaker of deionized water within the wet sample dispersion unit. Measurements were made in triplicates when obscurations of 10–15% were reached. Two refractive indices (RI) were used: 1.33 (water/background), and 1.42 (yolk/sample) (Kralik, Gajčević, Suchý, Straková, & Hanžek, 2009).

### **Protein surface hydrophobicity**

Protein surface hydrophobicity ( $S_o$ ) of the unfrozen and frozen yolk mixtures was determined using 1-anilino-8-naphthalene sulfonate (ANS) as a hydrophobic probe (Wu, Hettiarachchy, & Qi, 1998). The protein was serially diluted with deionized water to obtain protein concentrations ranging from 0.000675 to 0.01925%. Twenty microliters of ANS (8.0 mM in 0.1 M phosphate buffer, pH 7.0) were added to 4 mL of the diluted protein solution. The fluorescence intensity (FI) of the protein was measured in duplicates using Synergy™ H4 Microplate Reader (BioTek, Winooski, VT). Excitation and emission wavelengths were 390 and 470 nm, respectively. The FI reading was standardized by adjusting the spectrofluorometer reading for 10  $\mu$ L of ANS in 5 mL methanol to 80% of full scale. The slope of the plots of FI vs. percentage of protein concentration was calculated by least squares linear regression and used as the surface hydrophobicity.

### **Statistical analysis**

Statistical analysis was performed with JMP Pro 13, statistical software from Statistical Analysis System (SAS) Institute Inc. (Cary, NC). One-way analysis of variance (ANOVA) tests

were conducted, and significance of difference ( $p < 0.05$ ) was calculated using Tukey's HSD (honest significant difference) test.

## **Results and Discussion**

### **Effect of mechanical treatments on yolk gelation**

The effect of mechanical treatment such as rotor-stator high-speed mixing and colloid milling was tested by evaluating changes in hardness of freeze-thawed yolk and particle size distribution of processed yolk. Figure 3.1A and 3.1C show that applying the rotor-stator homogenizer to yolk at different speed for 90 seconds did not cause observable changes in the frozen-thawed yolk hardness and particle size distribution. Sirvente, Beaumal, Gaillard, Bialek, Hamm and Anton (2007) also found no change in yolk protein structure following rotor-stator treatment at 20,000 RPM for 90 seconds. Therefore, rotor-stator treatment at 8,000 RPM for 90 seconds was selected to achieve optimal mixing during the preparation of yolk mixed with additives.

According to Lopez et al. (1954), colloid milling of yolk at 0.003 inch for 3 passes is able to significantly decrease the viscosity of unfrozen egg yolk which also resulted in a lower viscosity gel when the yolk is frozen and thawed. Our result shows that colloid milling caused a reduction of hardness from 118.5 to 103.4 g (Figure 3.1B) and a shift in particle size distribution toward more abundance of smaller particles (Figure 3.1D). Protein aggregations occur during freezing, which was reflected through the shift of distribution towards larger particle size (data not shown). The fresh untreated yolk particles size ranged from 2.5-50  $\mu\text{m}$ , and colloid-milled yolk ranged from 0.1-40  $\mu\text{m}$ . Freezing caused a shift to larger particles ranging from 2.9-955  $\mu\text{m}$  for both untreated and colloid-milled yolk, but the abundance of larger particles in the colloid-



milled yolk was less compared to the untreated yolk. This shows that colloid-milling can help to reduce the degree gelation, although not to the extent of conventional gelation inhibitors.

### **Effect of different additives and their combinations on yolk gelation**

#### **Effect of different additives on hardness**

At 5% concentration, HCMC, HEW, HEY, proline, sugar and salt were effective in reducing gelation during short term 45 hour freezing (Figure 3.2A). Arginine and Tween 80 were also tested because they have shown ability to prevent protein aggregation elsewhere (Arakawa, Ejima, Tsumoto, Obeyama, Tanaka, Kita, et al., 2007; Hillgren, Evertsson, & Alden, 2002). However, it is important to note that egg yolk is a heterogeneous mixture of lipoproteins, and not just pure protein dispersion. These additives are not as effective in preventing lipoprotein aggregations in egg yolk.

Additives capable of better preventing gelation were further studied to determine the lowest concentration for their optimal gelation-inhibiting effect. Figure 3.2B shows a negative linear correlation between the amount of additive and yolk hardness, and 5% addition is the minimum concentration needed to produce good gelation inhibitory effect that is comparable to the industrially practiced 10% salt.

Tests were conducted to evaluate the effect of the different degree of hydrolysis of HCMC and hydrolyzed peptides. Our results showed that different MW of HCMC did not show a significant difference in gelation reduction (Figure B.1). Increasing the concentration of HCMC resulted in lower hardness, but this trend was not observed when HCMC concentration exceeds 7.5% (data not shown). The experiments for Figure B.1A and B.1B were conducted separately and different freezing storage time was used. Yolk with no additive frozen for 5 days

(Figure B.1A) formed harder gel than the yolk frozen for 1 day (Figure B.1B). This shows that yolk remains dynamic at -20°C and gelation occurs not only during freezing and thawing, but also during freezing storage (Au, 2015).

Hydrolyzed egg white and yolk proteins produced under different hydrolysis conditions did not show marked difference in gelation-inhibiting ability (data not shown). Addition of these peptides at 5% concentration achieved comparable gelation inhibition as 10% salt. Hardness was reduced from 93.02 g to 10.66, 10.11, and 16.53 g by 5% HEW, 5% HEY, and 10% salt, respectively.

Proline also proved to be a very effective gelation inhibitor. The hardness of proline-treated yolk was reduced by 87% compared to the yolk without additive. When added at 10% concentration, it inhibited gelation almost completely and the yolk maintained its fresh texture. However, proline is relatively expensive compared to salt and sugar, and the amount of proline as an additive is not allowed to exceed 4.2% of the total protein content in the food ("Food Additives Permitted for Direct Addition to Food for Human Consumption," 2017).

Sugar works similarly with proline. At 5% addition, gelation was reduced; but at 10% addition, gelation was completely inhibited and the yolk fluidity was preserved. Because of this, the hardness of 10% sugar yolk was not measurable, and 10% salt yolk was used for target comparison among the other additives.

No significant difference was observed in yolk gelation treated with 5% and 10% salt. Unlike other treatments, the yolk viscosity was markedly increased upon the addition of salt (Figure 3.2A), the yolk mixture was darker, more sticky and transparent. Based on this observation, it is apparent that each additive has different mechanism in reducing the degree of gelation.

### **Combination treatment and identification of synergistic effect**

Based on visual observation, HEW, HEY, proline and sugar seem to follow similar mechanism in inhibiting gelation. Although these additives were found effective in lowering the degree of gelation, HEW, HEY, and proline are relatively more expensive than salt or sugar. Preliminary test showed that unlike the other additives, addition of HCMC at high concentration had negative effect in hardness, and it was hypothesized that HCMC can prevent aggregation by forming electrostatic interactions with protein. Therefore, it is in our interest to determine if these additives could work synergistically with each other to inhibit gelation at lower concentrations, based on the unique gelation-inhibiting mechanism that each of these additives has.

Figure 3.3 shows the hardness of frozen-thawed yolk treated with different combinations of additives. Linear increase in hardness was observed as proline concentration is reduced and HCMC concentration is increased, indicating that proline and HCMC do not prevent gelation synergistically (Figure 3.3A). Similar trend is observed in treatments involving HCMC – HEY and HCMC – HEW (Figure 3.3B and C).

Interestingly, synergistic effect was observed between HCMC and sugar. The hardness of yolk containing 2.5% HCMC 2.5% sugar is significantly lower than those containing only 5% HCMC or 5% sugar (Figure 3.3D). This finding shows that HCMC has the potential to reduce the amount of sugar currently used to prevent gelation in commercial frozen yolk product.

Conversely, HCMC – salt showed counter-synergistic effect when used in combination (Figure 3.3E). The yolk gel was harder when the combined additive contains higher proportion of HCMC to salt. According to Pawlik and Laskowski (2004), CMC molecules coil with increasing ionic strength, making it less soluble in brine solutions. Therefore, HCMC might have

been inactivated and the gelation inhibition was mostly due to salt, as shown through the decreasing hardness with increasing salt content.

No difference was exhibited when salt and hydrolyzed peptides are used alone or in combinations (Figure 3.3F). Similarly, no synergistic effect was formed between sugar and hydrolyzed peptides (Figure 3.3G). The different trends observed from the effects of adding different additives confirmed that these additives have different gelation-inhibiting mechanisms, which are discussed more in depth in the next section.

### **Combination of additives with colloid milling**

Figure 3.4 shows how colloid milling affected gelation. The milling was able to significantly reduce gelation, and the addition of 2.5% proline 5% HCMC to the milled sample was effective to inhibit gelation comparable to the performance of 10% salt (Figure 3.4). Colloid milling was reported to decrease the degree of gelation of frozen yolk; the smaller the clearance of the mill, the less the gelation. The flavor, color, and texture of the colloid milled frozen-thawed yolk was very similar to those of fresh yolk after frying in Crisco (Lopez, Fellers, & Powrie, 1954). The same authors reported that salted colloid milled yolk had higher degree of gelation compared to the non-milled sample containing the same amount of sodium chloride. This, however, is not supported by our result, where the salted colloid-milled yolk has a significantly lower hardness than the non-milled salted yolk.

### **Mechanism of gelation inhibition by selected additives**

Gelation in frozen-thawed egg yolk is known to be caused by aggregations of proteins (Au, Acevedo, Wang, & Horner, 2015; Chang, Powrie, & Fennema, 1977; Hasiak, Vadehra, Baker, & Hood, 1972; Primacella, Acevedo, & Wang, 2017; Soliman & Van Den Berg, 1971).

Many factors can lead to protein aggregations, including concentration of yolk components and dehydration of LDL micelles due to formation of ice crystals, exposure of previously inaccessible hydrophobic regions due to change in protein structure when pH or ionic strength were altered, etc. While most researchers suggested plasma LDL as the main contributor to gelation, recent studies have shown that gelation involves a heterogeneous mix of aggregates including the major granule component HDL (Au, Acevedo, Wang, & Horner, 2015; Primacella, Acevedo, & Wang, 2017). We speculated that food additives that can interfere with ice crystal formation/growth or prevent protein-protein interactions will inhibit gelation. Gel strength measurement by itself does not provide sufficient information on how the additive prevents gelation. To better understand how each additive was able to successfully inhibit gelation, the changes in protein structure were monitored through measurement of particle size distribution, protein surface hydrophobicity, and amount of freezable water. The overall gelation-inhibition mechanisms of HCMC, HEW, HEY and proline are illustrated in Figure 3.5. Detailed explanation of each mechanism is discussed in the following sections.

### **Effect of salt**

Our results showed that the additives inhibited gelation through different mechanisms. The addition of salt caused a remarkable increase in the viscosity of the yolk mixture even before freezing, but the frozen-thawed yolk did not gel. Figure 3.6A showed a significant reduction in the particle size of both fresh and frozen-thawed salted yolk. Increasing the salt concentration from 5% to 10% increase the abundance of the smaller yolk particles. The surface hydrophobicity of the system was also shown to increase significantly, especially with the addition of 10% salt (Figure 3.6B). According to Wang, Li, Jiang, Qi and Zhou (2014), and increase in surface hydrophobicity can be caused by protein denaturation, dissociation, or

expansion of peptide chains, while formation of aggregates causes a decrease in surface hydrophobicity. Our finding shows that salt causes dissociation of proteins, altering the protein conformation and exposing more hydrophobic surface for ANS to bind. This is in agreement with previous finding where high level of salt can cause disruption of lipoproteins (Chang, Powrie, & Fennema, 1977; Kaewmanee, Benjakul, & Visessanguan, 2009; Lai, Chi, & Ko, 1999). According to Telis and Kieckbusch (1998), salt dissociates into ions when in solution, and these ions electrostatically shield proteins, increasing repulsion, which explains the increase in yolk viscosity when salt is added.

The melting/crystallization transition temperatures and amount of freezable water also significantly reduced (Figure 3.6C). Wakamatu et al. (1983) suggested that as an inhibitor of gelation in LDL solutions, NaCl increased the unfreezable water through formation of LDL-water-NaCl complex where water did not freeze. Chang, Powrie and Fennema (1977) found that salt only inhibited gelation when the yolk is frozen at a temperature higher than the eutectic temperature of coexisting salt. This suggested that LDL aggregation might be caused by progressive removal of water from LDL due to ice formation, and salt prevents this from happening by forming complexes with water and LDL.

### **Effect of sugar/sucrose**

Sucrose showed a completely different mechanism in inhibiting gelation. Its addition lowered the viscosity of the unfrozen yolk (data not shown) and 10% sucrose yolk was completely fluid. Sugars have been commonly used as stabilizers to protect proteins from degradation during lyophilization and storage. Two main hypotheses have been proposed to explain the stabilization mechanism of sugar: the “water substitution” hypothesis and the “glass dynamics” hypothesis. In the “water substitution” hypothesis, stabilizers are said to form

hydrogen bonds at specific sites on protein surface and thus substitute for the stabilization function of water that is lost during drying. The glass dynamic hypothesis suggests that sugar forms a rigid, inert matrix in which the protein is molecularly dispersed, limiting the mobility necessary for protein aggregation (Wang, Tchessalov, Warne, & Pikal, 2009). Lee and Timasheff (1981) also agreed that sucrose does not affect protein conformation. Its stabilizing mechanism is by increasing the free energy of the system while being preferentially excluded from the protein domain.

Our results are in agreement with this. There are no significant changes in protein size and hydrophobicity caused by the addition of sugar (Figure 3.6A and B), and the melting point and freezable water were reduced (Figure 3.6C). The changes in size and surface hydrophobicity at 10% addition are relatively small in the frozen-thawed sample compared to the unfrozen sample, showing that sugar is a very effective gelation inhibitor.

### **Effect of HCMC**

CMC, an anionic, water soluble polymer derived from cellulose, is widely used as food additive and is known to form charge-charge electrostatic complexes with proteins (Imeson, Watson, Mitchell, & Ledward, 1978). In egg yolk plasma, an interaction between the hydrocolloid CMC and the LDL is most likely caused by the negatively charged carboxyl groups of the CMC and the positively charged side chains of the amino acids in the LDL, leading to an agglomeration of the LDL micelles (Ulrichs & Ternes, 2010). For this study, CMC is partially hydrolyzed for the production of low viscosity, low molecular weight material that will not agglomerate the LDL due to the polymeric structure, but rather to prevent protein aggregation.

Figure 3.6A shows how HCMC affected the particle size distribution in fresh and frozen-thawed yolk. The measured yolk particles are relatively smaller compared to fresh yolk, and this

supports the statement earlier that HCMC forms electrostatic interaction with proteins, thus increasing the net negative charge and repulsive forces between proteins (Huan, Zhang, & Vardhanabhuti, 2016). Gelation still caused the distribution to shift toward larger particles, but not to the extent of untreated frozen-thawed yolk. Protein surface hydrophobicity test also confirmed this mechanism. HCMC-treated yolk had a significantly higher surface hydrophobicity than untreated yolk before freezing Figure 3.6B. The negative net charges were keep proteins apart in aqueous solution that made is easier for the hydrophobic ANS probe to access the hydrophobic region on the yolk proteins. The melting point and freezable water were also significantly reduced due to the high solubility of HCMC.

### **Effect of proline**

Amino acid proline has been reported to suppress aggregation during refolding of bovine carbonic anhydrase, egg white lysozyme, arginine kinase, creatine kinase and aminoacylase (Kim, Yan, & Zhou, 2006; Kumat, Samuel, Jayaraman, Srimathi, & Yu, 1998; Meng, Park, & Zhou, 2001; Samuel, Kumar, Ganesh, Jayaraman, Yang, Chang, et al., 2000; Xia, Park, Mu, Zhou, Wang, & Meng, 2007). Figure 3.6B showed how surface hydrophobicity was significantly reduced following the addition of proline, possibly because the previously available hydrophobic region has been bound to proline and no longer accessible to ANS. No significant change in protein size distribution was observed except that the smaller size population present in fresh untreated yolk is no longer present, and the size distribution becomes more uniform (Figure 3.6A). There was a slight shift towards larger particle size in the frozen-thawed sample, meaning that some aggregations still occur. The melting transition temperature and freezable water also decreased (Figure 3.6C).



According to Rudolph and Crowe (1986), proline forms hydrophobic stacking in aqueous solution through the formation of hydrogen bonding between the imino group of proline with the negatively charged carboxylate group of the adjacent proline molecule. The carboxyl groups of proline can also form hydrogen bonding with the solvent water molecules (Samuel, et al., 2000). This amphiphilic proline assembly suppresses aggregation by shielding the hydrophobic, aggregation prone region of the proteins (Kumat, Samuel, Jayaraman, Srimathi, & Yu, 1998; Schobert & Tschesche, 1978).

### **Effect of hydrolyzed proteins**

Hen egg white and egg yolk were enzymatically hydrolyzed using pepsin for production of short peptides. Enzymatic hydrolysis is known to increase the value of food proteins by modifying their physical and nutritional properties. Other than reducing MW, increasing the number of ionizable groups, and causing the exposure of hydrophobic groups, enzymatic hydrolysis improves the solubility of proteins and modulates their surface or interfacial properties such as stabilization of emulsions and foams (Foegeding & Davis, 2011). Hydrolysis of egg protein using pepsin has been shown to produce peptides with strong antioxidant activity and angiotensin I-converting enzyme (ACE) inhibitory activity (Davalos, Miguel, Bartolome, & Lopez-Fandino, 2004).

Based on our results, we speculate that peptides were able to inhibit gelation not only by preventing the growth of ice crystals, but also by hydrophobically shield the yolk proteins. The significant reduction in the surface hydrophobicity of yolk treated with both HEW and HEY (Figure 3.6B) suggested that less hydrophobic region was available on the protein surface, similar to the case of proline-treated yolk. The DSC results for both HEW and HEY (Figure 3.6C) also showed similarities to proline which suggest that these additives might have worked

following the same mechanism with proline. While proline as a food additive has a limit of 4% for safe consumption, HEW and HEY do not have a set limit. In fact, these additives can provide added value due to their high protein and low-fat contents.

Previous studies showed that peptide effectiveness in inhibiting crystal formation and growth is dependent on the size range and peptide source. Peptides in the MW of about 2-5 kDa from hydrolyzed gelatin were shown to be able to inhibit recrystallization of ice in ice cream mix (Damodaran, 2007). Peptides from collagen source inhibited ice recrystallization at MW range of 0.6-2.7 kDa (Wang & Damodaran, 2009). It is accepted that the inhibition mechanism involves binding of these peptides to the ice-liquid interface, which primarily involves hydrogen bonding. As measured by SDS-PAGE, the peptides produced from our egg white and yolk hydrolysis were no larger than 15 kDa.

### **Conclusion**

HEW, HEY, HCMC, and proline have proven effective in inhibiting gelation through different mechanisms. These additives can be used in combination with each other, sugar and colloid milling for further reduction in hardness. Future work is essential to determine the properties of the yolk products as important functional ingredient in food processing. The yolk performance as foaming, gelling and emulsifying agent can be evaluated, and sensory tests can be conducted to assess the acceptability of the yolk products. More optimization studies can also be performed to determine the most efficient hydrolysis condition for CMC and peptides, as well as best working concentration for each additive for prolonged freezing period.

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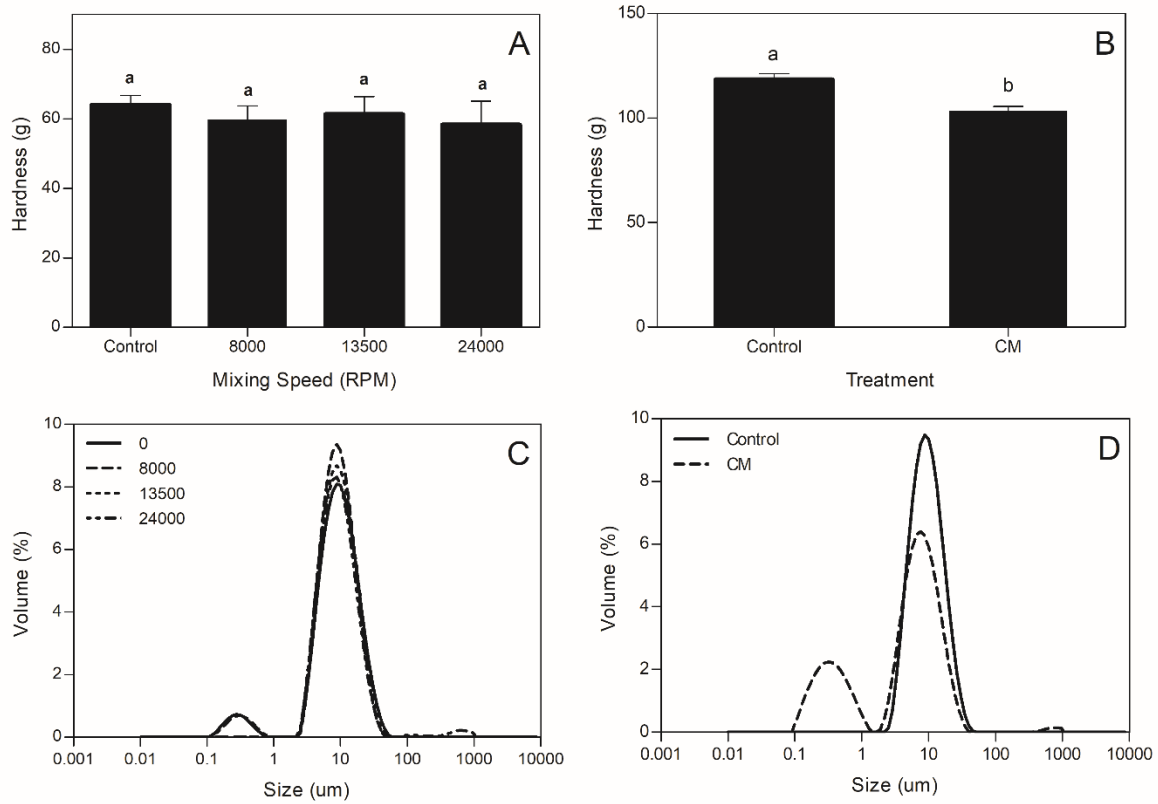
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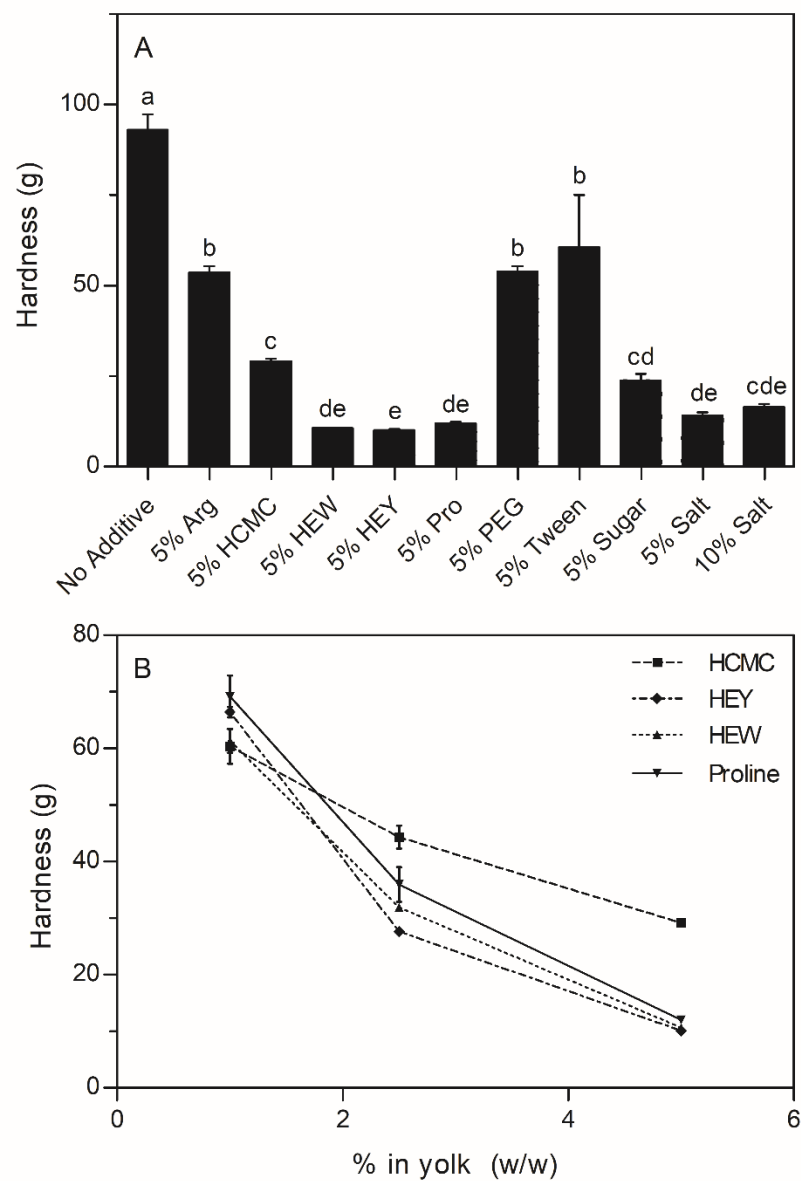
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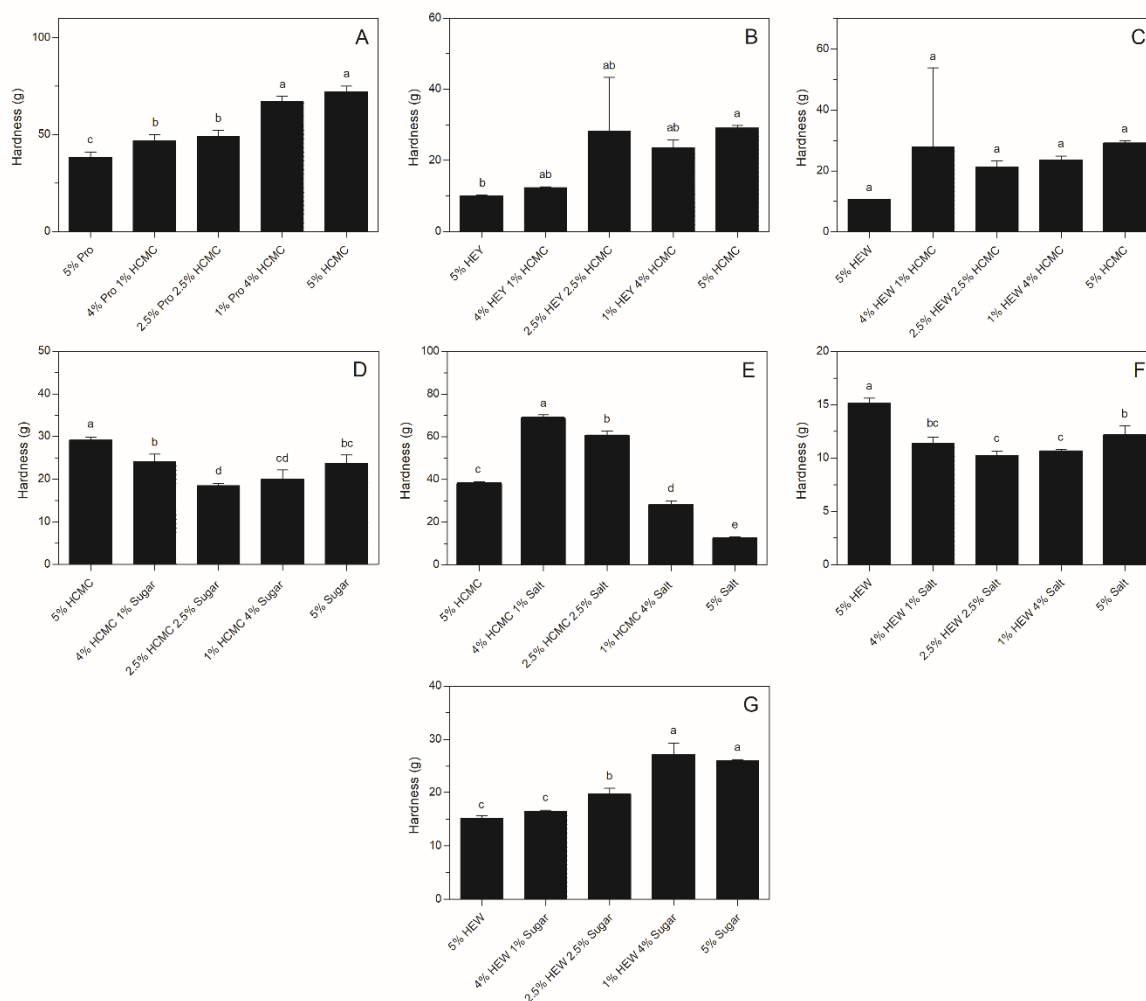


**Figure 3.1.** Effect of rotor-stator mixing speed (A, C) and colloid milling (B, D) on the hardness and particle size distribution of frozen-thawed yolk stored at  $-20^{\circ}\text{C}$  for 45 hours. Values with different letters are significantly different ( $p < 0.05$ ).

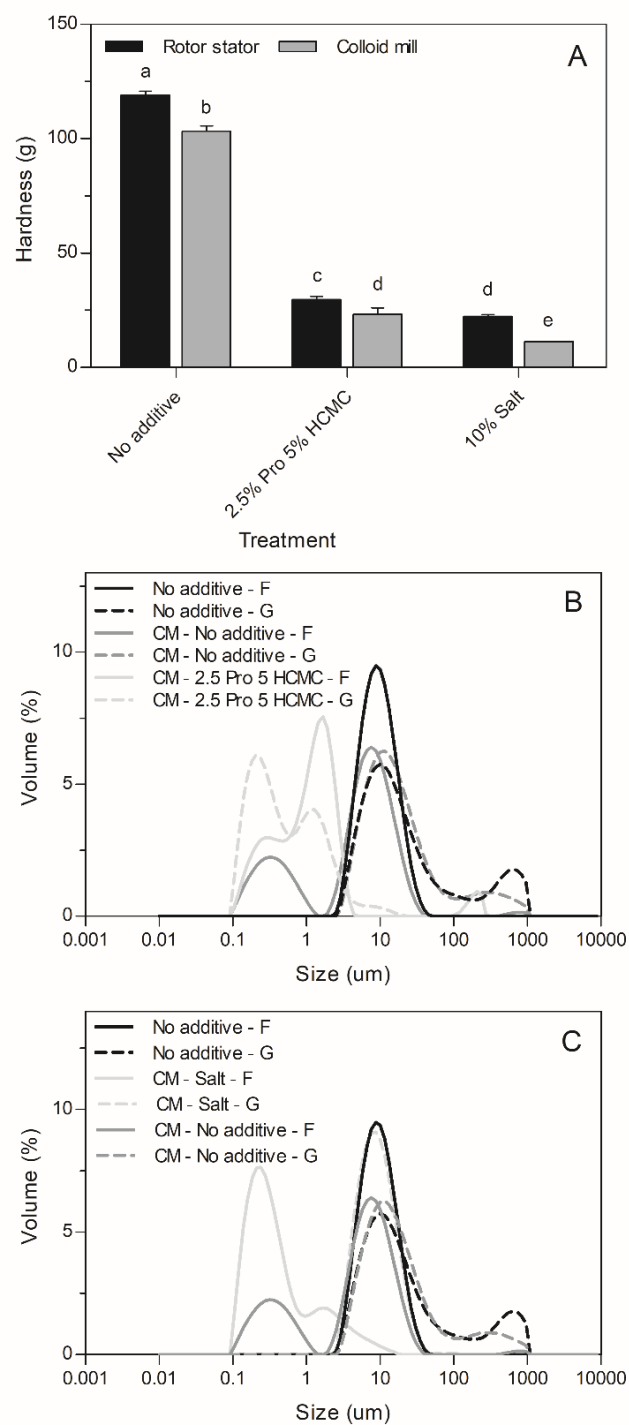


**Figure 3.2.** Effect of various additives (A) and quantity of additives (B) on hardness of frozen-thawed yolk (stored at  $-20^{\circ}\text{C}$  for 45 hours). Values with different letters are significantly different ( $p < 0.05$ ).

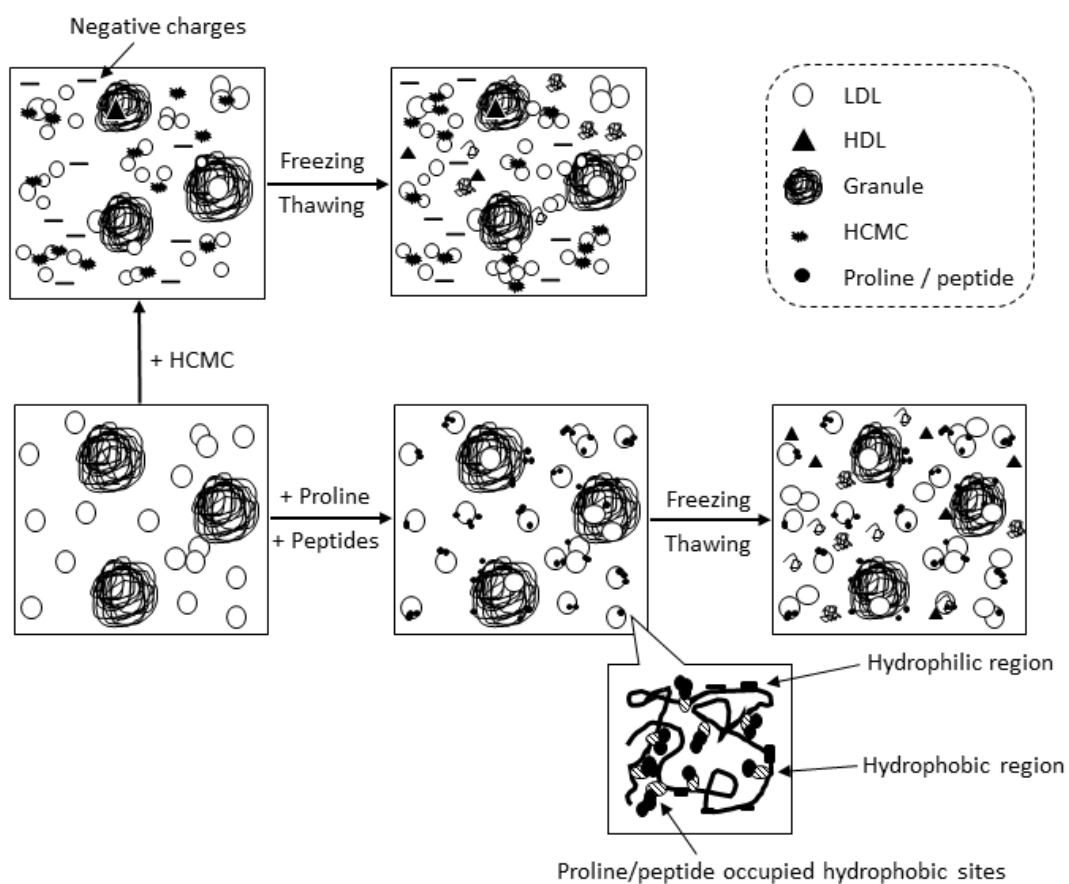




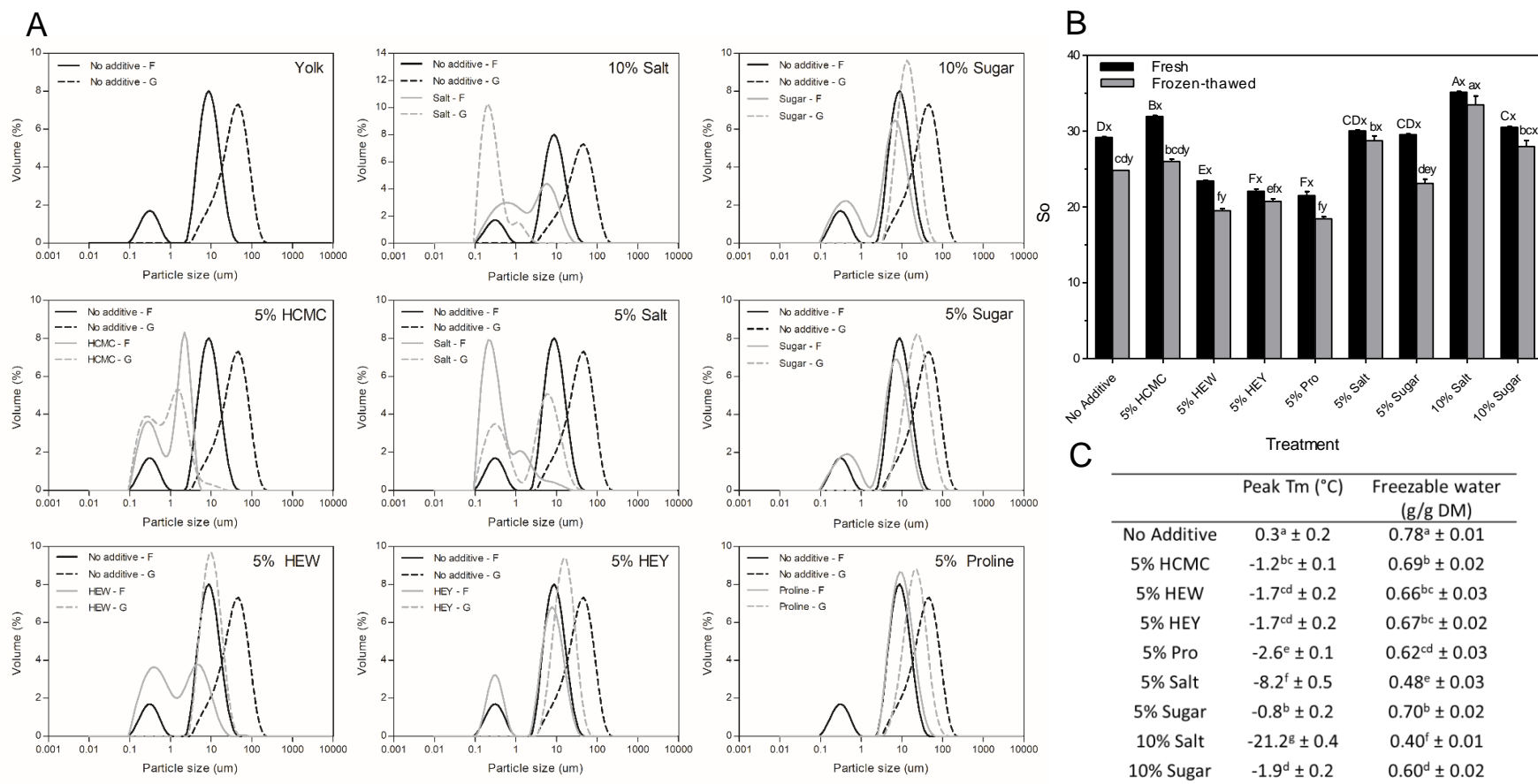
**Figure 3.3.** Hardness of frozen-thawed yolk (stored at  $-20^{\circ}\text{C}$  for 45 hours) treated with combinations of additives at 5% (w/w) concentration including Proline-HCMC (A), HEY-HCMC (B), HEW-HCMC (C), HCMC-sugar (D), HCMC-salt (E), HEW-salt (F), HEW-sugar (G). Values with different letters are significantly different ( $p < 0.05$ ).



**Figure 3.4.** Effect of colloid milling / additive on the hardness (A) and particle size distribution (B-C) of yolk frozen at  $-20^{\circ}\text{C}$  for 45 hours. Abbreviations are F, fresh; G, frozen-thawed; CM, colloid milled.



**Figure 3.5.** Proposed gelation-inhibiting mechanism by HCMC, proline, and peptide in frozen-thawed egg yolk



**Figure 3.6.** Particle size distribution (A), protein surface hydrophobicity (B), and amount of freezable water (C) of yolk containing various additives. Abbreviations are F, fresh yolk; G, frozen-thawed yolk (stored at -20°C for 45 hours).

## CHAPTER 4. GENERAL CONCLUSIONS

The overall objective of this research was to elucidate the mechanism of egg yolk gelation induced by freezing and thawing, and to identify treatments that can inhibit gelation. The first study (Chapter 2) focused on evaluating the changes in protein distribution upon 5-week freezing in yolk fractions obtained from recombined yolk systems containing different proportions of plasma and granules. The gel strength of the frozen-thawed yolk systems was also measured. Results showed that both plasma and granule components including LDL, HDL, and  $\alpha$ -livetin contribute to gelation through protein aggregation, which was reflected through the large mass increase in granule fraction and appearance of a floating LDL layer during fractionation of the frozen-thawed systems. Additionally, a significant increase in gel strength was observed in the yolk systems containing higher granule content. A detailed schematic presentation of yolk gelation mechanism was included in the end of this chapter. Overall, this study provides a better understanding of yolk gelation mechanism that may consequently lead to the design of innovative methods for preventing gelation.

In the 2<sup>nd</sup> study (Chapter 3), novel food additives and mechanical treatment that are effective in reducing gelation in frozen-thawed yolk (frozen for 45 hours) were identified. These include HPMC, HEW, HEY, proline, and colloid milling. The effectiveness of the food additives was assessed by comparing the hardness of frozen-thawed yolk containing these additives with those containing 5% salt and sugar. Hardness reduction of as high as 89.1% was achieved using one of the selected additives, as compared to 84.8% and 82.2% by 5% and 10% salt, respectively. Evaluation of the changes in the amount of freezable water, particle size distribution, and protein surface hydrophobicity in unfrozen and frozen yolk showed that these additives might inhibit gelation through different mechanisms, such as water binding,

electrostatic interaction, shielding of protein hydrophobic region, etc. For this reason, using combinations of additives paired with colloid milling might result in further reduction in the degree of gelation. Our results showed that yolk treated with HCMC and sugar had significantly lower hardness than those treated with HCMC or sugar alone. Combinations of other additives did not show as good synergistic effect, but they can still effectively prevent gelation.

Overall, this research showed that yolk gelation during freezing and thawing involves more than just plasma LDL aggregation, but also HDL and some livetin proteins. Better understanding of the gelation mechanism has led to more findings in treatments that can inhibit gelation, potentially replacing the currently used 10% salt or sugar. The suggested food additives have proven effective in reducing hardness, but future work is essential to determine the properties of such yolk products as important functional ingredient in food processing. The yolk performance as foaming, gelling and emulsifying agent should be evaluated, and sensory tests should be conducted to assess the acceptability of the yolk products. More optimization studies should also be performed to determine the most efficient hydrolysis condition for CMC and peptides, as well as best working concentration for each additive for prolonged freezing period.

## APPENDIX A. RELATIVE OPTICAL DENSITY ANALYSIS

**Table A.1.** Relative optical density (OD) analysis of HDL yolk protein of different molecular weights in granule and LDL fractions before and after gelation in all yolk systems

	1:0				1:0.5				1:1				1:2			
	Granule		LDL		Granule		LDL		Granule		LDL		Granule		LDL	
	Fresh	Gelled	Fresh	Gelled, floating	Fresh	Gelled	Fresh	Gelled, floating	Fresh	Gelled	Fresh	Gelled, floating	Fresh	Gelled	Fresh	Gelled, floating
<b>31</b>					11.9%	5.0%	0.9%	5.3%, 4.7%	9.3%	6.9%		3.2%, 3.5%	10.0%	8.7%		3.1%, 3.9%
<b>HDL 47</b>			1.5%	2.2%, 4.5%	6.7%	4.0%	1.7%	2.1%, 3.1%	5.1%	3.2%	1.8%	3.2%, 3.5%	4.5%	4.2%	1.4%	1.5%, 4.1%
<b>110</b>			1.0%	2.2%, 1.8%	22.8%	19.0%	0.9%	5.3%, 4.7%	14.1%	11.5%	1.8%	4.8%, 4.3%	14.9%	12.9%	1.8%	4.6%, 5.2%

**Table A.2.** Relative optical density (OD) analysis of LDL yolk protein of different molecular weights in livetin before gelation and LDL fractions before and after gelation in all yolk systems.

	1:0			1:0.5			1:1			1:2		
	Livetin	LDL		Livetin	LDL		Livetin	LDL		Livetin	LDL	
	Fresh	Fresh	Gelled, floating	Fresh	Fresh	Gelled, floating	Fresh	Fresh	Gelled, floating	Fresh	Fresh	Gelled, floating
<b>11</b>	6.2%	8.5%	8.2%, 7.2%	5.7%	12.0%	11.5%, 9.3%	5.9%	5.8%	5.2%, 5.15%	5.2%	7.7%	7.1%, 5.8%
<b>LDL 17</b>	4.5%	12.0%	11.2%, 9.0%	5.7%	13.8%	11.5%, 9.3%	3.8%	8.9%	7.5% 6.8%	3.7%	9.5%	7.1%, 6.6%
<b>93</b>	1.1%	2.5%	2.2%, 1.8%	0.9%	3.4%	2.1%, 1.6%	0.5%	2.5%	2.8%, 2.2%	0.4%	2.8%	2.0%, 1.9%
<b>122</b>	3.9%	8.5%	5.6%, 4.1%	3.4%	9.5%	5.3%, 3.9%	1.6%	5.8%	3.6%, 4.1%	1.5%	7.0%	3.1%, 3.9%

**Table A.3.** Relative optical density (OD) analysis of LDL yolk protein of different molecular weights in plasma fraction before and after gelation in all yolk systems.

		<b>1:0</b>		<b>1:0.5</b>		<b>1:1</b>		<b>1:2</b>	
		Fresh	Gelled	Fresh	Gelled	Fresh	Gelled	Fresh	Gelled
<b>LDL</b>	<b>11</b>	8.2%	6.6%	8.2%	5.6%	5.6%	2.8%	6.3%	1.6%
	<b>17</b>	8.8%	7.4%	8.2%	5.6%	5.2%	2.1%	5.1%	1.6%
	<b>93</b>	1.8%	0.8%	2.0%	0.9%	1.4%	na	1.1%	na
	<b>122</b>	4.7%	2.2%	5.1%	2.7%	2.4%	1.4%	2.3%	na

**Table A.4.** Relative optical density (OD) analysis of livetin proteins in livetin fraction before and after gelation in all yolk systems.

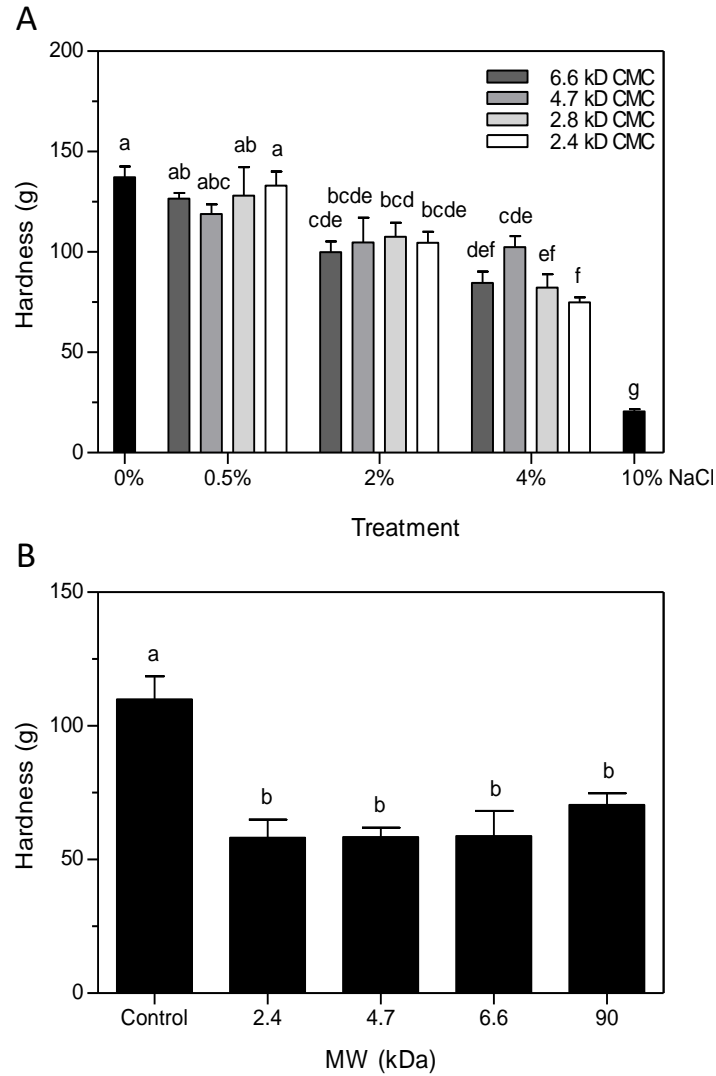
		<b>1:0</b>		<b>1:0.5</b>		<b>1:1</b>		<b>1:2</b>	
		Fresh	Gelled	Fresh	Gelled	Fresh	Gelled	Fresh	Gelled
<b>a-livetin</b>	55	5.6%	1.9%	8.0%	2.1%	3.8%	2.0%	3.7%	1.9%
	73	10.1%	10.6%	12.6%	12.0%	7.5%	7.4%	6.3%	5.8%
<b>b-livetin</b>	33	15.7%	29.8%	18.3%	40.9%	11.3%	16.2%	14.9%	19.8%
	36	5.6%	10.6%	8.0%	14.4%	6.5%	8.8%	5.6%	7.7%

**Table A.5.** Relative optical density (OD) analysis of LDL yolk protein of different molecular weights in granule fraction before and after gelation in all yolk systems.

	MW	<b>1:0</b>		<b>1:0.5</b>		<b>1:1</b>		<b>1:2</b>	
		Fresh	Gelled	Fresh	Gelled	Fresh	Gelled	Fresh	Gelled
<b>LDL</b>	11			5.2%	5.0%	3.8%	4.6%	4.5%	4.5%
	17			2.6%	5.0%	3.5%	4.1%	3.6%	4.2%



## APPENDIX B. EFFECT OF HYDROLYZED CARBOXYMETHYL CELLULOSE ON YOLK GELATION



**Figure B.1.** Hardness of frozen-thawed yolk treated with HCMC of different molecular weights, and frozen at -20°C for 5 days (A) and 1 day at 2% (w/w) concentration (B). Values with different letters are significantly different ( $p < 0.05$ ).